Modelling of risk in individuals with a possible genetic predisposition to pancreatic cancer

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine by

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Declaration

I declare that this thesis and the research upon which it is based is the result of my own work. Wherever I have incorporated the work of others, it has been clearly stated.

This work has not been submitted in any substance for any degree, nor is it concurrently being submitted in candidature for this or any other University.

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Abstract

At the time of writing this thesis, the overall 5 year survival from pancreatic cancer was 3%, mainly due to late presentation. The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer was established to identify individuals at high risk of pancreatic cancer and to offer them screening. At the time the work described in this thesis was initiated, relatively few individuals had been offered screening, which combined with the low number of prospective cancers in the registered high risk families made a reasonable diagnostic yield from screening unlikely.

The primary aim of this work was, therefore, to improve risk stratification on an individual basis for members of both hereditary pancreatitis and familial pancreatic cancer kindreds and to pilot a trial of secondary screening in individuals with an increased risk of pancreatic cancer.

The aims relating to risk stratification were met. Better primary screening led to increasingly in depth characterisation of high risk kindreds. A computer model was devised that calculated survival on an individual basis in familial pancreatic cancer and the possible utility of glucose to aid early diagnosis was investigated. In the hereditary pancreatitis kindreds, the clinical phenotype produced by the p.A16V mutation of the *PRSS1* gene was characterised for the first time. A multi-centre study of secondary screening for early pancreatic cancer in high risk individuals was also established, though at time of writing, the world's first curable pancreatic cancer has yet to be detected by screening.

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1 Introduction

1.1 Physiology of the Pancreas

The pancreas has two physiological functions, an exocrine function involved in digestion and an endocrine function relating to glucose metabolism. These actions are performed by different cells that operate as separate functional units. This section will focus on pathways leading to pancreatitis and the development of diabetes. As will be described later in the thesis, the development of diabetes could be a crucial element in identifying both early tumours and high risk individuals.

The majority of the pancreas is made up of lobules which contain multiple spherical clusters (acini) made up of acinar cells which are joined to ductal cells in a continuous epithelial layer. The intra-lobular ducts drain into a central intra-acinar terminal ductule. These contribute to progressively larger ducts until the main pancreatic duct of Wirsung and accessory duct of Santorini are formed, which empty into the duodenum[1].

The primary function of the ductal cells is to produce water and bicarbonate which neutralise the low pH of chyme. The role of the acinar cells is more complex. They produce pancreatic enzymes, which have a crucial role in digestion. The enzymes are produced within the acinar cells' rough endoplasmic reticulum and are transiently stored in an inactive form within zymogen granules[1]. They are released into the duodenum via the ductal system in response to acetylcholine and cholecystokinin, which are released by local nerves and the duodenal mucosa respectively. Once in

the duodenum, trypsinogen is converted to trypsin, triggering activation of the other pancreatic enzymes[1].

The exact aetiological process/processes leading to acute pancreatitis remain unclear but a general feature is thought to be either premature activation of trypsinogen to trypsin or the failure to eliminate active trypsin within the pancreas[2]. The triggers for this are most commonly gallstones and heavy alcohol consumption[3]. Chronic pancreatitis is characterised by fibrosis of the gland. It has been hotly debated whether this chronic disease is a completely separate condition from acute pancreatitis (in much the same way as cirrhosis of the liver and hepatitis are considered as separate entities) or whether this is just a natural progression from recurrent (possibly sub-clinical) acute attacks[4]. Most cases of chronic pancreatitis have traditionally been seen in those with a high intake of alcohol but there is an increasing understanding of the role of genetic factors in chronic pancreatitis[2], with mutations in, for example, the Cystic Fibrosis Transmembrane Receptor (*CFTR*) gene apparently increasing an individual's susceptibility[5, 6].

An understanding of Hereditary Pancreatitis (HP) is important as it will be discussed repeatedly in this thesis. There are 3 different types of human trypsinogen, termed *cationic*, *anionic* and *meso-trypsinogen*. These are coded for by the genes *PRSS1*, *PRSS2* and *PRSS3* respectively[7]. *PRSS1* mutations cause pancreatitis[8, 9], but it appears that *PRSS2* and *PRSS3* mutations do not[10-12].

There is still dispute and scope for further work on the biochemical effects of trypsinogen mutations, and it may prove to be the case that most reported 'mutations' actually have no effect and that they are actually artefactual[13]. In contrast

the main mutations seem to have pleiotropic effects *in vitro.* Sahin-Toth performed molecular analysis on recombinant trypsinogen with a range of mutations and showed that most of them, including the most common mutations, led to increased auto-activation of trypsinogen[7], but it is equally clear that the most common mutation p.R122H reduces self-inactivation of trypsin[14]. A novel mechanism, the 'Unfolded Protein Response'[15], was recently revealed in a p.R116C kindred. It is unclear which of these mechanisms has the greatest effect *in vivo.*

The endocrine function of the pancreas is performed by specialised cells that form the Islets of Langerhans. The human pancreas contains 1-2 million islets, predominantly within the body and tail, organised around small capillaries which act as the mechanism for release of the cell products into the systemic circulation[1].

The islets contain 5 known cell types. Alpha cells produce glucagon[1]; beta cells produce insulin[1] and islet amyloid polypeptide[16]; delta cells produce somatostatin[1]; and pancreatic polypeptide cells produce pancreatic polypeptide[16]. Epsilon cells were discovered in mice in 2004[17] and produce Ghrelin. The description of mechanisms of the different cell products will focus on those related to diabetes.

Insulin production by the beta cells is triggered by a complex series of feedback loops. The one relating to serum blood glucose is the best understood[18]. The main function of insulin is facilitating the storage of energy, whenever there is an excess of energy substances within the body. Excess carbohydrates are stored as glycogen within the liver and muscles, excess fats are stored in the adipose tissues and any carbohydrates that are unsuitable for glycogen production are converted to fats under the stimulus of insulin for storage[1]. Insulin directly promotes amino acid uptake

and conversion into protein as well as inhibiting the breakdown of proteins that have already been produced[1]. Glucagon, produced by the alpha cells, has several functions that are diametrically opposed to those of insulin. Glucagon's production is again closely related to serum blood glucose and it's principle effects on glucose metabolism are the promotion of glycogenolysis (which increases serum blood glucose within minutes) and the promotion of gluconeogenesis in the liver from amino acids[19]. Diabetes results from either inadequate production of insulin, which can result from pancreatic damage, or a reduction in the systemic response to glucose, which is the main pathway in the development of late onset diabetes in the obese[1].

1.2 Pancreatic Ductal Adenocarcinoma

The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). Other malignancies occur in and around the pancreas but for the purposes of this thesis, whenever the term pancreatic cancer is used, this will refer to PDAC unless explicitly specified.

Pancreatic cancer is the tenth most common malignancy in males but the fourth leading cause of cancer death in both men and women[20]. The latest published figures available for England and Wales are for the year 2000[21]. These show the incidence for the year 2000 to be 10.5/100,000 and 7.9/100,000 in males and females respectively, with 17,919 patients diagnosed between 1996 and 1999. Incidence is falling slightly in both sexes, with the gap between men and women reducing. These changes may be due to changes in smoking behaviour.

The only hope of cure is surgical resection but the high rate of locally advanced and metastatic disease at time of presentation means that surgical treatment is only an option for a small minority at the time they are diagnosed. The best estimates of the proportion of all those that are suitable for resection come from population based studies and varies from 2.6%[22], through 4.2%[23], 9%[24] to 13.4%[25]. The resection rate tends to be higher with more recent data[24, 25] and has been estimated to be as high as 15-20%[26], but definite calculations remain difficult.

If resection is possible, five year survival at a single centre has been reported to be as high as 32%[27]. Even better figures have been reported for early stage disease[28], but the results from the ESPAC-1 (European Study Group of Pancreatic Cancer 1)

trial[29] of 21% are more typical. Latest figures from the Mayo clinic give a five and ten year survival of 18% and 13% respectively[30], with five year survival at Johns Hopkins being 17%[31]. As only the minority of those affected can be treated surgically, overall survival remains poor, with 12 and 60 month survival for all those diagnosed in England and Wales being 12% and 2% respectively[21].

The general trend in post resection survival is improving[32] but the progress may not be as dramatic as some authors claim[33]. Progress is likely to be multi-factorial. Concentration of pancreatic services within tertiary centres will have contributed[34-36], and trial evidence has helped to clarify aspects of clinical care[29]. More radical surgery has not been shown to make a significant difference to long term survival[37].

This thesis is based on the assumption that screening (and so earlier surgery) will offer better survival. It is therefore of great importance to note that post-operative chemotherapy has shown survival benefit in the ESPAC-1 trial, a large randomised controlled trial (RCT), although there was no discernable benefit in a chemo-radiotherapy group[29]. A second large RCT also showed no survival benefit from adjuvant chemo-radiotherapy[38]. This suggests that poor survival results from a systemic disease that is at least partially treatable. It can be reasonably hypothesised that the metastatic burden will at least be lower with earlier detection and so easier to treat. The findings of ESPAC-1 have been accepted in Europe but have not been universally accepted in the US[26] where chemo-radiotherapy remains in use.

The chemotherapeutic agent used in ESPAC-1 was Fluorouracil (5-FU) and this remains in widespread use, delivered orally as the pro-drug Capecitabine. The

ESPAC 3 trial has shown no survival difference between 5-FU and Gemcitabine in the adjuvant setting[39] although Gemcitabine is now the established agent of choice when chemotherapy is being used with palliative intent[40]. Combinations of agents are an area of ongoing research, particularly in the palliative setting. The addition of cisplatin to gemcitabine has been shown to be beneficial in advanced biliary tract carcinomas[41] but this benefit was not shown in advanced pancreatic cancer[42]. The combination of capecitabine with gemcitabine showed a significant increase in both the objective response rate and progression-free survival and was associated with a trend toward improved overall survival compared with Gemcitabine alone[43]. Chemo-radiotherapy has also been considered pre-operatively[44] and agents such as the epidermal growth factor receptor inhibitor, Erlotinib, have become available although their role remains limited[45]. One major question which still needs to be addressed is whether there is a role for neo-adjuvant treatment in pancreatic cancer, although the answer is likely to be some years away.

1.2.1 Types of Pancreatic Lesion and the PanIN Model

This thesis will propose that in screening for pancreatic cancer in high risk groups the ideal result will be the identification of precancerous lesions (in preference to identification of adenocarcinoma). The arguments for this will be made later, but it is necessary to first understand the nature of these lesions.

In 2000 a progression model was proposed where normal pancreatic ductal cells developed through an adenoma-carcinoma sequence in a transition towards malignancy[46]. There was already good evidence for this in colorectal cancer[47] but it took several years to achieve an acceptance of a similar pathway for pancreatic cancer. The classification system for pancreatic lesions that was first proposed in 1994[48] was built upon in 1999[49] when pancreatic intraepithelial neoplasia (or PanIN) was split into three sub-groups; PanIN 1 (divided into types A and B), PanIN 2 and PanIN 3. Definitions were tightened in 2003[50] but the underlying system was not altered and the definitions have been increasingly accepted with time.

Normal tissue was defined as having normal ductal epithelium, ranging from cuboidal to low-columnar epithelium with amphophilic cytoplasm and no evidence of mucinous cytoplasm, nuclear crowding or atypia. The next step beyond normal is squamous (transitional) metaplasia, which was defined as the replacement of normal cuboidal ductal epithelium by mature stratified squamous or pseudo-stratified transitional epithelium, in the absence of cytological atypia[50].

PanIN-1A lesions were defined as flat epithelial lesions comprising tall columnar cells with basally located nuclei and abundant supranuclear mucin. They have small nuclei which are round-to-oval in shape. If oval, these are oriented perpendicular to

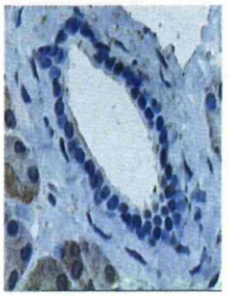
the basement membrane. The difference between a PanIN-1a and a PanIN-1b was defined as the presence of papillary, micropapillary, or basally pseudostratified architecture[50].

PanIN-2 lesions were defined as mucinous epithelial lesions that can be flat but are mostly papillary, with some nuclear abnormalities. These abnormalities might include loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification, and hyperchromatism, but falling short of the abnormalities seen in PanIN-3. Mitoses are rare, but when present are non-luminal and are not atypical[50].

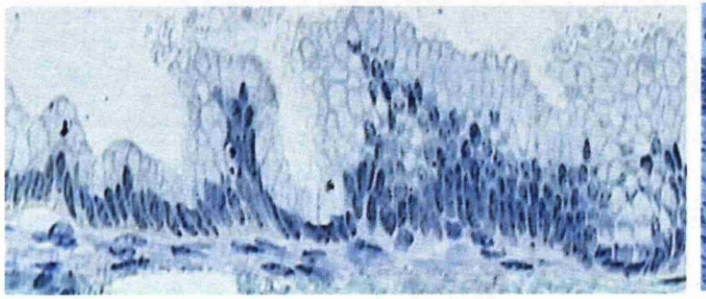
A diagnosis of PanIN 3 was suggested by true cribriforming, the appearance of 'budding off' of small clusters of epithelial cells into the lumen and luminal necrosis. The lesions are generally papillary or micro-papillary, though they can still be flat. Cytologically, PanIN 3 lesions have lost nuclear polarity, with the presence of dystrophic goblet cells, mitoses, nuclear irregularities, and prominent (macro) nucleoli. Overall, cellular appearances were in keeping with carcinoma, but without invasion of the basement membrane[50]. These changes are presented visually in figure one.

Embyro

Pdx-1
Sonic **h**edge-**h**og (SHH)
Serine/**t**hreonine **k**inase **11** (STK11/LKB1)



Benign ductal cells



PanIN-1

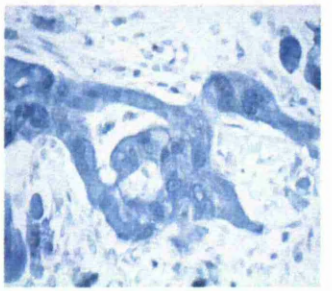
K-ras
Telomere shortening
p21 (WAF1/CIP1)
Human **e**pidermal growth factor **r**eceptor **2** (HER2/neu)
Mucin **1** (MUC1)
MUC 6
Trefoil **f**actor **1** (TFF1)
p16 (INK4a)
S100A11
MUC5AC
S100A6

PanIN-2

Cyclin D1
Cyclo-**ox**ygenase-**2** (COX-2)
Hes1 **(H**air and **e**nchancer-**o**f **s**plit **1)**
Notch 1
Pepsinogen C
Kruppel-like **f**actor **4** (KLF4)
HOXA5
GATA5
Gastrin
Villin-1
Villin-2
Cellular **r**etinoic **a**cid **b**inding **p**rotein **1** (CRABP1)

PanIN-3

p53
SMAD4
BRCA2
S100P
SHH
SialyT (Mucin-associated-carbohydrate antigen)
Maspin
MUC4
Tumour **s**uppressor in-**l**ung **c**ancer-**1** (TSLC-1)



Invasive carcinoma

Figure 1: Histological images of benign pancreatic ductal epithelial cells, progressive PanIN lesions and invasive carcinoma, with associated genetic alterations.

Reprinted (with consent) from P Ghaneh *et al* (2007), Gut 56 (8) 1134-52[51].

Figure illustrates a progression from left to right from normal ductal epithelial tissue through to invasive adenocarcinoma, through a series of histologically defined precursor lesions. The genetic mutations associated with each stage are also shown.

The evidence to support the theory that PanINs are the precursors of invasive pancreatic adenocarcinoma results from large pathological studies, case studies and identification of genetic markers.

Pathological evidence includes the original study by Cubilla and Fitzgerald[52], who analysed more than 1000 pancreatic specimens. They identified histologically distinct proliferative lesions in pancreatic ducts and ductules adjacent to infiltrating pancreatic adenocarcinoma. The lesions were initially dubbed 'hyperplasias' and were shown to be more common in the specimens with cancer than in those without. A similar study, Kozuka *et al*[53], corroborated the findings. PanINs are more common in the pancreatic head and increase in frequency with age. Luttges and Kloppel[54] pointed out that this is the same pattern seen in pancreatic ductal adenocarcinoma.

Additional evidence was provided by Brat *et al*[55], where three cases of infiltrating ductal adenocarcinoma were reported 17 months to 10 years after the identification of atypical papillary duct lesions within the pancreas. Similarly, Brockie *et al*[56] described two patients with atypical papillary lesions who subsequently developed invasive pancreatic ductal adenocarcinoma.

The genetic analysis of PanINs provides strong evidence of a link to adenocarcinoma and generally supports the progression model. The genetic mutations associated with each stage of the PanIN model have been shown above in figure one[51]. There are well established genetic abnormalities associated with PanINs on the *K-RAS2* gene (which codes for the K-Ras oncoprotein)[57-61] and the tumour suppressors *CDKN2A* (which codes for p16)[62, 63] and *Tp53* (which codes for

p53)[61, 64]. Mutations of these three genes are all tested for as part of EUROPACs secondary screening study outlined later in this thesis. As figure one shows, there are a large number of other PanIN associated gene mutations affecting members of the ErbB family of receptor tyrosine kinases such as HER1 and HER2)[61, 65], other tumour suppressor genes such as SMAD4[66], and caretaker gene mutations such as BRCA2[67]. In addition to gene mutations, other genetic factors associated with PanIN lesions include telomere shortening[68], aberrations in cell cycle control mechanisms such as over-expression of $p21^{WAF/CIP1}$[69], growth factor signalling changes such as over-expression of COX-2 and inappropriate activation of embryonic signalling pathways such as Hedgehog[70, 71] and Notch[71].

It is widely accepted that pancreatic cancers can also develop from precursor lesions other than PanINs. These include both mucinous cystic neoplasms (MCN), and intraductal pancreatic mucinous neoplasms (IPMN). The definitions relating to IPMN were defined in a WHO publication in 2000[72], and the associated genetic changes are becoming increasingly well characterised[73-79]. In time this may permit clinicians to differentiate between mucinous lesions that will follow a benign course and those that will undergo malignant change.

1.2.2 Symptoms and Signs of Pancreatic Cancer

The symptoms and signs of pancreatic cancer are relevant to this thesis as although they are generally non-specific and normally occur, as stated already, when the cancer is already incurable. They certainly play a part in any vigilance system and at present represent the bench mark against which any screening system must be judged. In particular diabetes will be discussed in this thesis as it may, in time, open new opportunities for screening and earlier diagnosis.

The classical presentation is of epigastric pain which radiates to the back, weight loss, and painless obstructive jaundice[80]. Pain originating from a pancreatic tumour can be felt anywhere in the T6 to T10 dermatomes. It is initially difficult to localise, although it can be more pronounced in body and tail tumours and is the most common presenting complaint in larger tumours[81]. Weight loss is often a relatively late symptom and is more common in tumours of the pancreatic head. A proportion of the weight loss associated with pancreatic cancer will be due to exocrine pancreatic failure and the associated reduction in absorption of nutritional intake, but this will be exacerbated by increased metabolic requirements of malignant cells as the primary grows and metastatic disease progresses. The weight loss associated with malignant disease accelerates as the tumour mass increases. Obstructive jaundice can be the trigger that leads to presentation in those with potentially resectable disease[81]. Should the tumour develop in the pancreatic head (the commonest site for pancreatic cancer), obstruction of the biliary tree may occur relatively early and trigger diagnosis, whilst the tumour is still potentially curable.

Occasionally individuals may experience other symptoms. An estimated 1% of newly diagnosed diabetics aged greater than 50 years have developed diabetes as a direct result of an emerging pancreatic cancer[82] and there may be non-specific symptoms such as nausea and vomiting. Nausea and loose stools could develop as a result of exocrine pancreatic failure. Gastric outlet obstruction would obviously lead to vomiting, but this would normally be expected to only occur in those with locally advanced disease[83].

By far the most common physical sign associated with potentially curable pancreatic cancer is jaundice[84]. Less commonly, one may see left supra-clavicular lymphadenopathy (Troisier's sign), or other signs associated with jaundice, liver failure or portal venous hypertension such as pruritus, spider naevi or caput medusae respectively.

Attempts have been made to characterise the symptoms and signs associated in pancreatic cancer, but except for those related to cholestasis, these are of limited clinical application[84, 85].

1.2.3 Diagnosis

The investigative process may be triggered by the presence of the symptoms or signs described in the previous section or, as cross sectional imaging becomes increasingly common, more pancreatic cancers are being diagnosed incidentally[86]. Logically, a greater proportion of individuals with these tumours will be potentially curable as the tumour has been detected before clinical signs and symptoms have become apparent. The most common initial investigations if a patient presents and pancreatic cancer is suspected is serum CA19-9 and a computed tomography (CT) scan of the abdomen. If the results of either of these tests showed evidence of a pancreatic tumour, in the United Kingdom (UK) at least, the case would be referred to a multi disciplinary team (MDT) meeting. The outcome of the discussion at this meeting would be: referral to a resectional centre; palliative oncology after the confirmation of the diagnosis; or best supportive care, if the patient was not a candidate for either of the first two treatment modalities.

1.2.4 Current Treatment

All potential resections in the UK are put through the supra-regional MDT meetings at the relevant regional centre. Once all appropriate investigations have been performed and cytological evidence obtained wherever possible, a decision is taken as to what the most likely diagnosis is and the most appropriate treatment. For pancreatic cancer, the key decision is whether the tumour is resectable. This is guided by the degree of local invasion and the presence of metastatic disease. At the Royal Liverpool University Hospital, which is the centre which performs the pancreatic surgery for patients from Merseyside and much of North Wales, this decision is informed by the CA19-9 level. If the CA19-9 is greater than 150 kU/L, a laparoscopic ultrasound is performed[87]. This is a more sensitive method of assessing vascular encasement, looking for small liver metastases and finding small peritoneal deposits than staging CT. Those that still have potentially curable disease are subsequently listed for resectional surgery.

Those that have unresectable disease on presentation, or are shown to have incurable disease after either laparoscopy or laparotomy, are put forward for palliative chemotherapy. Palliative surgery or stenting is also performed as appropriate.

1.2.5 Prognosis

There has been some progress in one year survival of pancreatic cancer. This has been a result of regionalisation of services and the addition of adjuvant chemotherapy to surgical resection, but with only 10-15% of pancreatic cancers being suitable for surgical cure at presentation, overall five year survival remains in the region of 2-3%[20]. The low 5 year survival rate makes it imperative that pancreatic cancers are detected earlier in their developmental process, when they are still potentially curable. One of the problems with earlier detection by screening is that the sensitivity and specificity of the available imaging modalities is reduced, altering the balance of potential risk and benefit. The incidence of pancreatic cancer in the general population is low. As the specificity of the available imaging modalities falls, it becomes inevitable that the number of false positives increases. At some (as yet undetermined) point, there will be a crossover between the morbidity and mortality that is involved in operating on the inevitable false positives that result from screening and the lives saved by the earlier detection of pancreatic cancer. This is a difficult and as yet unresolved problem, but one way of optimising the risk to benefit ratio is to optimise the necessary screening protocols to groups at greatest risk of pancreatic cancer.

1.3 The High Risk Groups

Relatives of pancreatic cancer patients have an elevated risk of pancreatic cancer. This risk is mostly accounted for by a few families with multiple cases[88], with an estimated 5-10% of pancreatic cancer cases occurring as part of familial cancer syndromes associated with known mutations[89]. A recent meta-analysis shows the relative risk of a family history to be 1.80 (95% CI 1.48, 2.12), although the heterogeneity of the studies limited the sub-group analysis[90].

The cause of clustering of cancer cases within families will vary. The cancers may result from an inherited predisposition to a cancer associated condition such as hereditary pancreatitis[91-93] or diabetes[82]. Other families can be shown to carry known pancreatic cancer associated genetic mutations, such as *CDKN2A*[94, 95]; whilst others have been classified as belonging to the group *Familial Pancreatic Cancer* (FPC), where the causative mutation for the vast majority of families remains unknown[96, 97]. As this thesis will largely focus upon FPC, this will be the first of the groups to be discussed.

1.3.1 Familial Pancreatic Cancer

Familial Pancreatic Cancer (FPC) is the name given to the cancer syndrome where multiple cases of pancreatic cancer are clustered within a single family. Such clusters of cancers were originally thought to be due to common environment or coincidence, but seminal work by Henry Lynch and co-workers (initially with colorectal cancer) resulted in the gradual acceptance of a genetically determined predisposition with the first cohort of pancreatic cancer families presented in 1989[98]. The potential implications of genetic predisposition prompted the establishment of registries around the world, including the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC), with which the author is associated.

The term FPC is used to describe multiple cases of pancreatic cancer within families in a pattern consistent with autosomal dominant inheritance. This definition has gradually been strengthened by the registries to exclude families that belong to other cancer syndromes, which carry a predisposition to pancreatic cancer (e.g. Breast-Ovarian Syndrome), or hereditary illnesses such as Hereditary Pancreatitis (HP), which carry an associated increased risk of pancreatic cancer[99].

The causative gene in Familial Pancreatic Cancer (FPC) kindreds remains unidentified but segregation analysis of families on registries suggests a rare major gene conferring predisposition[100, 101]. Autosomal dominant transmission is not universally accepted but is the most likely form of transmission given a single major gene. An inherited predisposition for cancer is usually the result of a heterozygous

defect in a tumour suppressor, with loss of the second copy of the tumour suppressor being the second 'hit'. Genetic instability is part of the ageing process and so the second hit will be inevitable if an individual lives long enough.

1.3.1.1 The Evidence for FPC

Epidemiology

The sheer number of families that are included in registries provides strong evidence pointing towards FPC as a genuine genetically defined syndrome, with EUROPAC's figures shown in the first part of the results section of his thesis. At the time of data guillotine for this thesis, EUROPAC had registered more than 170 families with an apparent autosomal dominant predisposition to pancreatic cancer, with scores of other families where there are multiple cases of pancreatic cancer and no evidence for another genetic cause. Some of these families could be explained by confounding factors which will be described in section 1.3.1.2, equally the inclusion of some artefactual families cannot be ruled out, but the rigorous evidence required to meet the strict inclusion criteria may result in omission of genuine families, meaning the incidence of the syndrome may well be an underestimate.

Although, on average, age of onset is similar to that seen in sporadic disease, one phenomenon that has been discovered is 'anticipation' [102, 103]. In simple terms, the age of onset of pancreatic cancer within FPC families occurs at an increasingly young age in consecutive generations. The fact that average age of onset remains consistent with the sporadic disease is explained by later age of onset in earlier

generations balancing out the younger onset in their offspring. This could be explained by various forms of bias, but meticulous statistical analysis suggests that the phenomenon is real[101].

Genetics

Identification of the gene responsible for FPC requires a mutation that segregates with the disease. For most genetic syndromes linkage analysis has been used to identify such mutations, but FPC presents particular problems when applying such an approach. Pancreatic cancer is a late onset disease making it difficult to distinguish a carrier who is yet to develop cancer, from a family member who is not carrying the mutation. Ethical and logistical reasons make it impractical to obtain samples from every family member prior to an individual developing the disease. Once a family member is diagnosed, there is only a very short window of opportunity for research groups to approach patients for DNA, at a time of great stress for those affected. This makes conventional linkage studies extremely difficult and as a consequence most work has concentrated on candidate genes. Various candidate genes have been suggested, but these have either been found not to be mutated in FPC kindreds (such as *STK11*[104], *RNaseI*[105] and various Fanconi anaemia genes[106]), or they are only associated with pancreatic cancer as part of more general cancer syndromes (such as *CDKN2A*[94] and mismatch repair genes[107]). The only exception has been a small number of families which are entirely consistent with FPC and which carry *BRCA2*[108] or *PALB2* stop mutations[109], which have recently been shown to be associated with hereditary pancreatic cancer. It is noteworthy that

the protein product of *BRCA2*[110] has been shown to be a binding partner for the PALB2 protein[111].

The lack of progress made using candidate genes has prompted a return, despite its problems, to conventional linkage analysis and association studies. To overcome the problem of identifying carriers, Brentnall and colleagues used a surrogate of pancreatic dysplasia for pancreatic cancer. Patients with dysplasia were identified by screening within 'Family X', a large family characterised by a high incidence of diabetes as well as pancreatic cancer. Using this approach they were able to identify a region at the end of chromosome 4 which gave two point LOD scores of greater than 3, with three point LOD scores reaching a maximum value of 5.36[112]. A LOD score (logarithm (base 10) of odds) is a statistical test often used for linkage analysis and compares the likelihood of obtaining the test data if two loci are linked, to the likelihood of observing the same data by chance alone. The minimum defined area in Family X was 4q32-34 and the same group have now provided evidence that the disease mutation for this family lies within the *Palladin* gene[113].

The other FPC registries (which will be discussed in section 1.5.1 of this thesis) assessed their own families and showed that it was not just unlikely that the 4q32-34 locus accounts for a significant proportion of their registered FPC families[114, 115], but that the *Palladin* mutation was also absent in these kindreds[116-118]. Work is ongoing in a number of institutions, exploiting novel mathematical models to account for the ambiguity in defining carrier status[101] and new technology, such as SNP arrays, to increase the efficiency of linkage and association studies[119].

1.3.1.2 Arguments Against FPC:

There are a number of arguments that have been put forward criticising and even questioning the existence of FPC as a true genetic syndrome. The arguments have been discussed in full in a recent review of FPC[96] and are summarised below.

Misclassification and Chance

The overall lifetime risk of developing pancreatic cancer for the general population is 0.5-1%[120]. It is, therefore, not inconceivable that large kindreds (with scores of at risk individuals) could have two cases by chance alone.

Selection Bias

Pancreatic cancer patients in the USA were asked to report any other cases in first degree relatives. Approximately one in ten were able to do so[103, 121]. In these studies all the families will include at least one pancreatic cancer case (the proband). This means that the chance of two cases in one of these families is roughly equivalent to the chance of finding a single case in an unselected kindred. Case-control studies are desirable and these are discussed in more detail in the review[96].

Genetic Factors

The relative influence of genes and environment is a notoriously difficult area. People who share common genetic backgrounds often have similar diets, occupations and customs. Pancreatic cancer has been shown to be more common

in black than white Americans[122]; this could be due to low penetrance or multi-gene susceptibility, or simply that black Americans lead a lifestyle that is more 'high risk'. In support of an environmental rather than genetic link, migration studies show that pancreatic cancer risk amongst Japanese migrants moving to the US increases and overtakes the level of cancer risk of white Americans[123]. The most likely cause of this is the Japanese adopting the 'Western' high meat, high fat diet, although a direct link between Western diet and pancreatic cancer has not been proven, despite large cohort studies[124]. An indirect link via obesity and diabetes (see below) cannot be ruled out, but neither is there any evidence that it explains the migration studies.

Gender

Analysis of the Surveillance Epidemiology and End Results (SEER) data[120] shows a slightly greater incidence of pancreatic cancer in men than women in all age groups. The situation in high risk groups is less well understood and EUROPAC data and the SEER data were compared as part of the analysis of primary screening. The findings can be seen in figure 14 in the results section of this thesis.

Environmental and Lifestyle Factors

The best evidence for a link between an environmental risk factor and incidence of pancreatic cancer exists for tobacco smoking[125]. Overall, smoking increases the risk of pancreatic cancer by two-fold[126], with some evidence for a dose-response relationship[127]. The risk posed by passive smoking remains unproven, thus clustering of pancreatic cancer within families is more likely to be related to a

common habit shared by family members, than contamination of the family home by a single heavy smoker. Analysis of the EUROPAC database has shown no direct evidence for smoking as the cause of familial clusters of pancreatic cancer[101].

A link has been shown between pancreatic cancer and obesity[128]. Obesity shows familial clustering, thought to be due to shared behaviours, so this may contribute to some cases classified as FPC.

There has been particular emphasis on searching for a link between pancreatic cancer and occupations that lead to contact with chlorinated hydrocarbons, especially dichlorodiphenyltrichloroethane (DDT), though, again, no definite link has been established [129, 130].

Diabetes and Other Associated Medical Conditions

There are two major illnesses linked to pancreatic cancer; diabetes mellitus and chronic pancreatitis. Some 80% of pancreatic cancer patients have impaired glucose metabolism. Tumours can induce production of diabetogenic peptides which result in insulin resistance reminiscent of type 2 diabetes[131], which can often be alleviated by resection of the tumour[132]. In sporadic disease, development of higher baseline fasting glucose levels appears to be a very early symptom of pancreatic cancer[133] but this has not been shown in familial pancreatic cancer patients. It is also possible that diabetes is a risk factor, as well as a symptom, of pancreatic cancer but this remains unproven[132]. Diabetes shows familial clustering and as stated previously is a feature of Family X, one of the best characterised of all FPC families[112]. It is possible that diabetes could explain some cases classified as FPC on the EUROPAC database, but an analysis has failed to show an increased incidence of diabetes mellitus, above that expected as a symptom of pancreatic cancer.

An additional possible cause of artefactual familial clusters of pancreatic cancer could be multiple cases of chronic pancreatitis within a family. This could be caused by a shared tendency to heavy alcohol intake or the rare genetic syndrome, hereditary pancreatitis. Chronic pancreatitis has been shown to lead to a 15% lifetime risk of pancreatic cancer[134] and the cumulative lifetime risk increases to 35-53% in hereditary pancreatitis families[91-93], but given that EUROPAC also registers families with hereditary pancreatitis (HP) and most of the responsible mutations can be tested for, this is unlikely to be relevant in EUROPAC's case.

Interaction of Genetic and Environmental Factors

It is conceivable that multiple cases of pancreatic cancer in a family could be caused by genetic variations other than the elusive FPC mutation that could possibly increase the impact of environmental factors. Such variations, although inherited, would not justify the description of FPC, as the link is indirect. Genetic polymorphisms have already been linked to the development of pancreatic cancer and pancreatic adenocarcinoma has been shown to be associated with the UGT1A7*3 allele of UDP-glucuronosyltransferase, an enzyme known to be involved in detoxifying tobacco carcinogens[135], however, with >90% of the population having a very small risk of pancreatic cancer, it is unlikely that any commonly occurring polymorphism could cause a sufficient increase in risk to account for FPC. A rare combination of multiple unlinked polymorphisms should not lead to a family history of pancreatic cancer covering more than one generation.

Cystic Fibrosis (CF) affects multiple systems by causing obstruction of ducts; one organ affected is the pancreas. Two early onset cases of pancreatic cancer were identified in 28,000 cases of cystic fibrosis (odds ratio 31.5 vs. control group)[136] and there is a greatly increased risk of chronic pancreatitis[5, 6] in those with cystic fibrosis transmembrane receptor (*CFTR*) mutations. It is at least conceivable that a similar autosomal dominant inheritance of pancreatic cancer risk could be observed either under certain circumstances, or with specific *CFTR* mutations. A study of 166 early onset pancreatic cancer patients (under the age of 60) found 14 carriers of disease related *CFTR* mutations (8.4%) compared to 4.1% in controls (odds ratio 2.18, 95%

CI: 1.24–3.29)[137], but none of the 14 cancer patients had a family history of pancreatic cancer, which is unsurprising given the fairly modest increased risk.

Autosomal dominant inheritance of a predisposition to other forms of cancer is well known, for example colorectal cancer in hereditary non polyposis colorectal cancer or breast cancer in breast ovarian syndrome. It is conceivable, that by chance, a family with a more general syndrome could present with more than one pancreatic cancer case in the absence of other tumours. It should also be understood that although FPC is defined specifically in terms of pancreatic ductal adenocarcinoma, it is possible that ampullary tumours, extra-hepatic cholangiocarcinomas, acinar cell tumours and even pancreatic neuroendocrine tumours may have been included due to misdiagnosis. It is even possible that misdiagnosis, or misreporting of colorectal or gastric tumours could explain part of a cluster.

Other Cancer Syndromes that Predispose to Pancreatic Cancer

Registries such as EUROPAC, NFPTR and FaPaCa (described in full in section 1.5.1) require reliable evidence of pancreatic ductal adenocarcinoma before registering a family. This means that high penetrant syndromes with known disease mutations are unlikely to be confused with FPC. For example, mutations in the *VHL* gene which cause von Hippel-Lindau syndrome are associated with pancreatic neuroendocrine tumours[138], with only occasional pancreatic ductal adenocarcinomas reported in these families[139]. Li-Fraumeni Syndrome is associated with *Tp53* and *CHK2* mutations. At least 24 families have been reported with multiple cases of pancreatic cancer, which, superficially, would be consistent with FPC[140]. However, in

the same study the families were followed for 10 years and over 200 cases of non-pancreatic cancer were reported[140]. It is unlikely that such an extreme cancer risk would be missed by even the most cursory family analysis and so such families would not be included as FPC by any of the large registries. Another example, is Peutz-Jeghers syndrome (PJS); the autosomal dominant inheritance of hamartomatous polyposis. The reported increased risk for pancreatic cancer is very great (132-fold)[141] which is sufficiently high to produce a phenotype with multiple cases of pancreatic cancer within a family, but in the largest study of PJS to date, only 6 pancreatic cancer patients were reported. The reason for the small number of cases is the high mortality from other cancers in these families, so as for Li-Fraumeni it is very unlikely that a PJS family would be mistaken for FPC[142, 143]. EUROPAC originally had a policy of screening possible FPC families for the *STK11* mutations that cause PJS, but no mutations were identified[104].

Similarly, low penetrant cancer syndromes associated with well defined phenotypes other than cancer would be unlikely to be confused with FPC. For example, mutations in the *ATM* gene cause ataxia–telangiectasia, an autosomal recessive inherited disease characterised by oculocutaneous telangiectasias, cerebellar ataxia, and cellular and humoral immune deficiencies. People with ataxia-telangiectasia have increased cancer risk, estimated at 50 to 150-fold, but this would clearly be a recessive risk. Heterozygotes for ATM mutations have an approximately 3-fold increase in risk[144]. The specific risk for pancreatic cancer is, at most, marginal[145]. It is unlikely that such a low increased risk would give many familial clusters of pancreatic cancer and even if this did occur, a familial history of ataxia would be likely. Familial

adenomatous polyposis (FAP), which is caused by a mutation of the tumour suppressor gene *APC*, is characterised by the presence of multiple adenomatous polyps within the gastrointestinal tract. The colon is the most commonly affected site and there is a high incidence of colonic cancer. The elevation in risk of pancreatic cancer is relatively small, 4.46 (95% CI: 1.2-11.4) or 21.4 cases per 100,000 person years[146]. Although it is possible that a family would contain multiple cases of pancreatic cancer, due to the numbers of colonic cases, an FAP family would be unlikely to be diagnosed as a FPC kindred.

Although the majority of cancer syndromes are unlikely to be confused with FPC by major registries, there appears to be heterogeneity in the phenotype associated with certain mutations. For example, Hereditary Non-Polyposis Colorectal Cancer (HNPCC) can be divided into two groups (Lynch syndromes I and II). Both syndromes result from mutations in mismatch repair genes but Lynch syndrome I is almost exclusively associated with colorectal cancer, whilst Lynch syndrome II features extra-colonic tumours in sites such as the stomach, breasts, uterus, bladder and small bowel. This second group shows a clearly elevated risk for pancreatic cancer[147]. Another example is the phenotype that can result from mutations of the *BRCA*2 gene. This can lead to an autosomal recessive syndrome associated with lymphomas and hepatomas (Fanconi's Anaemia). In most cases these families have no noticeable increased risk for pancreatic or breast cancer[148]. In other families *BRCA*2 mutations are associated with autosomal dominant predisposition to breast and ovarian cancer[149], with other families having an autosomal dominant predisposition towards pancreatic cancer without an elevated risk of breast cancer.

The latter example includes families that have been defined as FPC[108]. Mutation of the *CDKN2A (Ink4a^{p16})* gene is associated with multiple naevi and cases of melanoma, a syndrome known as Familial Atypical Multiple Mole Melanoma (FAMMM)[150]. In other *CDKN2A* families there are also one or more cases of pancreatic cancer, this has been described as a separate syndrome (FAMMM-PC, OMIM #606719). To date, all FAMMM-PC families have included cases of melanoma, hence the probability of confusion with FPC is low. Testing of genuine FPC families has yet to identify any *CDKN2A* mutations[94]. A summary of the genes with germline variants associated with pancreatic cancer is presented below in table one, which is taken (with permission) from a review written by my MD supervisors[97].

Table 1: Genes with germline variants associated with pancreatic cancer

[a]Loss of function means that the germline mutation stops an allele working, leading to dependence on the other allele for the gene function.
Gain of function means that the mutation has a phenotype even when present in a heterozygote with a wild type copy of the gene.
Polymorphism means that the variant associated with cancer is present in a substantial proportion of the population with no overt phenotype, but may cause a subtle decrease (or increase) in the efficiency of the gene.
[b]Proteins often have multiple functions, this list just describes a category which best fits each gene. DNA repair is described in basic terms, recombination is meant to include any mechanism used to repair double strand breaks, NER is nucleotide excision repair and BER is base excision repair.
[c]Syndromes are defined clinically, some syndromes result from more than one mutation and some mutations are causative of more than one syndrome. Syndromes are autosomal dominant unless otherwise stated.
[d]Principal cancers other than pancreatic ductal adenocarcinoma linked to germ line variants in the gene.
[e]Where cancer risk is associated with another disease state and the other disease is associated with the variant in the gene.
[f]Monogenic if at least one family has been shown to have cancer predisposition that segregates with the mutation. Polygenic if the variant is associated with apparently sporadic disease or with cancer in only a single generation.
[g]Defined as independent unless the association with a genetic variant is only seen in combination with specific environmental exposures.

Gene	Nature of mutation[a]	Role[b]	Syndrome[c]	Other cancers[d]	Associated condition[e]	Strength[f]	Environmental Dependence[g]
BRCA2[108]	Loss of function	DNA repair (Recombination)b	Breast Ovarian FPC	Breast, ovarian, prostate	Direct	Monogenic	Independent
Palladin[113]	Gain of function	Motility	FPC with endocrine failure	None	Direct?	Monogenic	Independent
MLH1, MSH2, MSH6[151]	Loss of function	DNA repair (mismatch)	HNPCC	Colorectal	Direct	Monogenic	Independent
BRCA1[152]	Loss of function	DNA repair (Recombination)	Breast Ovarian	Breast, ovarian	Direct	Monogenic	Independent
CDKN2A (*p16*)[95]	Loss of function	Tumour suppressor	FAMMM	Melanoma	Direct	Monogenic	Independent
STK11[153]	Loss of function	Tumour suppressor	Peutz-Jeghers	Various gastrointestinal	Direct	Monogenic	Independent
APC[154]	Loss of function	Tumour suppressor	FAP	Colorectal	Direct	Monogenic	Independent
T*p53*[155]	Loss of function	Tumour suppressor	Li-Fraumeni	Various	Direct	Monogenic	Independent
PRSS1[92] (p.R122H/p.N29I)	Gain of function	Protease	Hereditary pancreatitis	None	Pancreatitis	Monogenic	Independent
FANCC[156]	Loss of function	DNA repair (Recombination)	Fanconi's Anaemia (Recessive)	Leukaemia, head and neck, oesopahgeal[157]	Direct	Polygenic	Independent?
MGMT[158]	Polymorphism	DNA repair (Base modification)	None	Colorectal (minor allele protective)[159]	Direct	Polygenic	Independent?
XRCC 1,2,3[158, 160]	Polymorphism	DNA Repair (Recombination)	None	Head and neck, lung	Direct	Polygenic	Smoking?[130]
APE1[158]	Polymorphism	DNA Repair (BER)b	None	No independent link – possible multigene dependence[161]	Direct	Polygenic	Independent?
XPD[162]	Polymorphism	DNA Repair (NER)b	Xeroderma pigmentosum (Recessive)	Skin, lung[163]	Direct	Polygenic	Independent?
CYP1A1[164]	Polymorphism	Xenobiotic	None	Lung, liver, oesophageal[165]	Direct	Polygenic	Smoking
CYP1A2[166]	Polymorphism	Xenobiotic	None	Lung, liver[167]	Direct	Polygenic	Smoking
NAT 1[166]	Polymorphism	Xenobiotic	None	Myeloma, lung, bladder[168]	Direct	Polygenic	Smoking

Gene	Nature of mutation[a]	Role[b]	Syndrome[c]	Other cancers[d]	Associated condition[e]	Strength[f]	Environmental Dependence[g]
MTHFR[169]	Polymorphism	Folate metabolism	Homocystinuria (Recessive)	Colorectal, leukaemia[170]	Direct	Polygenic	Independent?
MTRR[171]	Polymorphism	Folate metabolism	None related to cancer risk	Colorectal, leukaemia?[172]	Direct	Polygenic	Independent?
FasL[173]	Polymorphism	Apoptosis (Intercellular signalling)	None	Oesophageal[174]	Direct	Polygenic	Independent?
Caspase8[173]	Polymorphism	Apoptosis (Intracellular signalling)	None	Glioma[175]	Direct	Polygenic	Independent?
PRSS1[92] (p.A16V)	?	Protease	Hereditary pancreatitis?	None	Pancreatitis	Polygenic?	Independent?
PSTI(SPINK1)[176]	Polymorphism	Protease inhibitor	None	None	Pancreatitis	Polygenic	Independent?
CFTR[5]	Polymorphism	Chloride channel	Cystic fibrosis (Recessive)	Gastrointestinal[136]	Pancreatitis	Polygenic	Independent?
KCNQ1[177]	Polymorphism	Potassium channel	None	None	Diabetes mellitus (Type 2)	Polygenic	Independent?
HLA-A/B[178]	Polymorphism	Immune response	None related to cancer risk	Various infection related cancers[179]	Diabetes mellitus (Type 1)	Polygenic	Independent?
UGT1A7[135] *Disputed*[180]	Polymorphism	Xenobiotic	None	Disputed[180]	Pancreatitis?	Polygenic	Independent?
TNF-α[181]	Polymorphism	Cytokine	None	Gastric and other infection related cancers[182]	Pancreatitis?	Polygenic	Independent?
RANTES(CCL5[181]*]*	Polymorphism	Cytokine	None	Gastric and other infection related cancers[183]	Pancreatitis?	Polygenic	Independent?
CCR5[181]	Polymorphism	Cytokine	None	Cervical (HPV related)?	Pancreatitis?	Polygenic	Smoking

1.3.1.3 The Cancer Risk in FPC Kindreds

The definition of FPC as an autosomal dominant condition suggests that risk is equivalent to penetrance; however, this is complicated by the issues of classification (as discussed above) and the lack of a recognised disease mutation in most families. It is assumed that penetrance in FPC is high, but less than 100%. If penetrance in FPC were 80% to 75 years, then lifetime risk for a mutation carrier would be 80%. The risk to an individual in the same family without a mutation would be that of the general population (0.5-1%)[184]. In the absence of a test for mutation status in most families, the lifetime risk can only be estimated on the basis of some form of probability calculation giving the perceived chance that the individual is a mutation carrier. For example, half of all first degree relatives of pancreatic cancer patients in a genuine FPC family would be mutation carriers; on the basis of 80% penetrance they would therefore be estimated to have a 40% lifetime risk. On the discovery of a disease mutation, the estimation of risk for these same individuals would rise to 80% or fall to that of the general population depending on whether the individual was shown to be a gene mutation carrier. This does not take into account the possibility that the family only appears to be a FPC kindred.

An attempt at risk quantification was performed by Klein *et al*[185]. A prospective registry-based analysis showed that members of families with one confirmed pancreatic cancer death had a 4.6-fold increase in risk over the general population. If there were two confirmed cases the risk increased to 6.4-fold and was increased 32-fold in families with three affected members. Ignoring low penetrance conditions, this

equates to estimation of the likelihood that an individual is a member of a FPC family. Despite the obvious weaknesses that could be levelled at these calculations, these were the best data available on risk within FPC kindreds at the start of my period of research. The risk in EUROPAC's FPC kindreds is shown in figure 13 in the results section of this thesis.

1.4 Hereditary Pancreatitis

Hereditary Pancreatitis (HP) was first described in 1952 by Comfort and Steinberg[186] but it was not until 1996 that Whitcomb *et al*[8] isolated the first mutation in the cationic trypsinogen gene (*PRSS1*) on the long arm of chromosome seven (*7q35*). It is an autosomal dominant disease with penetrance that is generally accepted to be ≈80%[186, 187] and further work on this will be presented in the results section of this thesis. HP is characterised by frequent attacks of epigastric pain, which is normally associated with nausea and vomiting. Symptoms may start shortly after birth but onset varies greatly, with some individuals not exhibiting symptoms until adulthood. There is usually progression to chronic pancreatitis with endocrine and exocrine failure and an increased risk of pancreatic cancer[91-93]. The natural history of HP follows a similar pattern to alcohol associated chronic pancreatitis, but there are important differences, for example, HP has an earlier age of onset of pancreatitis although malabsorption and diabetes mellitus occur at a later stage in the disease[91-93].

Families are defined as having HP if the phenotype is consistent with highly penetrant autosomal dominant inheritance. In simple terms, this would require two or more first degree relatives (or three or more second degree relatives) to have unexplained recurrent-acute or chronic pancreatitis in two or more generations and this is the definition that has been adopted by EUROPAC[92].

The vast majority of the cases of HP are caused by mutations of the cationic trypsinogen gene (*PRSS1*), which lies on the long arm of chromosome seven.

Substitutions, at base 365 (c.365G>A) and base 86 of the cDNA (c.86A>T), were discovered in the late 1990s by conventional linkage analysis[8, 9]. They are now known as p.R122H[8] and p.N29I[9] according to the amino acid substitution and position in the protein sequence.

These mutations are rarely identified in general screens of patients with idiopathic disease[188-191] and the phenotype of p.R122H and p.N29I is now well characterised[91-93]. There are many other rare mutations or polymorphisms of *PRSS1* that are less well understood[192] and not all HP families have had the responsible genetic mutation identified. EUROPAC calls families with a phenotype consistent with HP, but no identified mutation (after sequencing of *PRSS1*), 'Neg All HP'. Their presence implies that there is at least one further mutation to be identified.

As stated in section 1.1, the mechanism by which these genetic mutations cause pancreatitis is incompletely understood but it is thought to be due to increased autoactivation[7] or reduced deactivation of trypsinogen, or a combination of the two.

1.4.1 Diabetes in Hereditary Pancreatitis

This subject has been the focus of much recent work by the EUROPAC study group. Data were analysed from 750 individuals (614 affected and 136 obligate carriers) from 145 families. There were a total of 191 individuals that developed endocrine pancreatic failure, with a total of 37 cancer cases, 35 in affected individuals and 2 in unaffected carriers. A Cox proportional hazard model showed that diabetes was an independent risk factor for pancreatic cancer, allowing for familial clustering using a gamma shared-frailty model, hazard ratio of 2.9 (95% confidence intervals of 1.47, 5.85). In short, diabetes was shown to be a risk factor rather than a symptom of cancer in most patients (Greenhalf *et al*, unpublished).

1.4.2 The Cancer Risk in Hereditary Pancreatitis Kindreds

A diagnosis of hereditary pancreatitis carries a substantial cancer risk. Lifetime risk has been variously calculated as 35-54% [91-93] to the age of 75 years. This is sufficient to merit secondary screening but the overall risk of pancreatic cancer in those affected by HP in the EUROPAC population is 4-5 times less than the cancer risk in FPC families as will be shown in figures 13 and 21 of this thesis.

1.4.3 Non-Causative Pancreatitis Associated Mutations

Both p.R122H and p.N29I are high penetrance mutations. Other genetic mutations have been identified which have been shown to have an associative rather than a causative relationship with pancreatitis. The two most common will be discussed below.

1.4.3.1 Cystic Fibrosis Transmembrane Receptor Mutations

As previously mentioned cystic fibrosis (CF) is an autosomal recessive condition with an incidence in the Caucasian population of 1:2500 live births. The causative gene, the cystic fibrosis transmembrane receptor (*CFTR*) gene, was located in 1989{Kerem, 1989 #35333}. The most striking effects (normally pulmonary) are seen in homozygotes, though 1–2% of homozygotes have been shown to develop chronic pancreatitis[193, 194]. Interestingly this predisposition to pancreatitis is also seen in heterozygotes. There are >1500 known mutations, although only a handful have been proven to be associated with pancreatitis. Much of this work has been performed in the past decade[6, 195, 196], with the most common mutation found to be p.F508del which comprises 66% of all mutated alleles[197]. The resultant phenotype can vary greatly from young onset idiopathic chronic pancreatitis with complete exocrine and endocrine pancreatic failure to later onset disease and almost normal pancreatic function, even for those with the same mutation.

The exact pathway by which *CFTR* mutations cause chronic pancreatitis is incompletely understood[5, 198-202]. The presence of a mutation does not inevitably lead

to chronic pancreatitis, suggesting either an environmental trigger, or that *CFTR* mutations contribute to a multigenic predisposition to pancreatitis. It has been argued that compound heterozygous *CFTR* mutation carriers have an even greater risk of chronic pancreatitis, which is increased further if a *SPINK1* mutation is also present[5, 200].

The Cancer Risk in *CFTR* Mutation Associated Chronic Pancreatitis

The degree of the cancer risk in *CFTR* related chronic pancreatitis is also incompletely understood. The overall lifetime risk of pancreatic cancer in individuals with chronic pancreatitis has been calculated at 15%[134]. There are insufficient data available to say whether the cancer risk in *CFTR* mutation associated chronic pancreatitis is greater or less than this. As stated previously, two early onset cases of pancreatic cancer have been identified in 28,000 cases of cystic fibrosis (odds ratio 31.5 vs. control group)[136] and a study of 166 early onset pancreatic cancer patients (under the age of 60) found 14 carriers of disease related *CFTR* mutations (8.4%) compared to 4.1% in controls (odds ratio 2.18, 95% CI: 1.24–3.29)[137], although none of the 14 cancer patients had a family history of pancreatic cancer. At present, individuals with *CFTR* mutation associated chronic pancreatitis are not screened for early pancreatic cancer as the risk of cancer is deemed too low to merit the risk of experimental screening.

1.4.3.2 SPINK1 Mutations

SPINK1 is a protease inhibitor which is thought to inactivate intra-pancreatic trypsin. This occurs by the formation of a covalent bond between SPINK1's carboxyl group and the catalytic serine residue of trypsin. Despite recent progress[203], the exact mechanism by which the most common mutation (p.N34S) causes chronic pancreatitis remains unresolved. Mutations in the Kazal type 1 serine protease inhibitor gene (*SPINK1*)[204-213] have been shown to be associated with pancreatitis, with the p.N34S variant present in over 20% of idiopathic patients[208, 210]. Twenty seven percent of Indian alcohol-related chronic pancreatitis patients have also been shown to carry p.N34S mutations[205], although it is not considered a causative mutation, being present in 1–2% of healthy controls.

The Cancer Risk in *SPINK1* Mutation Associated Chronic Pancreatitis

It has been shown that p.N34S mutations of *SPINK1* are not found in idiopathic pancreatic cancers[214] but the cancer risk in those with *SPINK1* mutation associated chronic pancreatitis is again poorly characterised and cannot be differentiated from the cancer risk in a cohort of chronic pancreatitis patients of all aetiologies[134]. Individuals with *SPINK1* mutation related chronic pancreatitis do not fulfil the inclusion criteria for the screening studies.

1.4.3.3 The p.A16V Mutation of *PRSS1*

One of the more significant steps from the work that went towards this thesis is the characterisation of the clinical phenotype produced by the third most common *PRSS1* mutation on the EUROPAC registry. A cytosine (C) to thymine (T) missense mutation in exon 2 that leads to an alanine (GCC) to valine (GTC) substitution at codon 16[191] is more simply known as p.A16V.

p.A16V is significantly associated with pancreatitis[191] and was first identified in pancreatitics with no family history[191]. It has subsequently been reported by other groups in apparently idiopathic patients[188] and is relatively rare in families with multiple cases of pancreatitis[92, 188, 191]. In contrast, rare instances of p.R122H in individuals without a family history may be explained by either a limited pedigree or by spontaneous mutation[215].

Whilst p.R122H and p.N29I have both been linked to increased autoactivation (or reduced deactivation) of cationic trypsinogen{Sahin-Toth, 2000 #2054;Sahin-Toth, 2000 #1707}, p.A16V lies at the edge of the signal peptide of trypsinogen and has previously been considered to influence secretion{Witt, 1999 #1605}. Secretion failure is still considered to explain the link between the p.R116C mutation of *PRSS1* and pancreatitis, but p.A16V mutant protein has been reported to be secreted normally{Kereszturi, 2009 #32374}. Other work has established that p.A16V increases the rate of chymotrypsin C (CTRC) activation of trypsinogen by

approximately four-fold. This results in accelerated trypsinogen activation *in vitro*, possibly explaining the link with pancreatitis{Nemoda, 2006 #14447}.

In 1999 Witt *et al*[191] published the first report of p.A16V in a paper detailing the results of genetic testing of children with chronic pancreatitis. Of the 44 children tested, 30 had apparent sporadic disease with a further 14 having a family history. Of the 30 sporadic cases, p.A16V was detected in three individuals. It was also detected in one individual with a family history. One p.R122H mutation was also detected in the family history group. Further individuals from families with p.A16V were tested for the mutation, with just one of seven carriers being affected. This suggested that p.A16V is a low penetrance mutation, although the numbers involved were too low for firm conclusions to be drawn.

In contrast to Witt's initial paper, EUROPAC has held data for many years on several families with p.A16V mutations and phenotypes consistent with HP, which would be indistinguishable from those of p.R122H or p.N29I. Prior to this thesis, p.A16V was poorly characterised in the literature and EUROPAC and the other registries held too few data in isolation for a meaningful analysis. It remained unclear whether p.A16V was a HP causative mutation in the same way as p.R122H and p.N29I or whether it was a pancreatitis associated mutation producing a similar phenotype to mutations in the *CFTR* and *SPINK1* genes. The results from the multi-centre collaborative project relating to p.A16V will be presented in Chapter 3.

The Cancer Risk in p.A16V Kindreds

The cancer risk due to chronic pancreatitis in p.A16V kindreds is uncertain. The occurrence of a single cancer case in a p.A16V family has already been published[92] but the data were too few for definitive conclusions and at the start of this thesis p.A16V pancreatitics were not entered into the trials of secondary screening.

1.5 Primary Screening and Traditional Methods of Risk Stratification

Screening can be thought of as primary or secondary. Primary screening is the identification of individuals at known high risk of an event, e.g. identification of families with a genetic predisposition to pancreatic cancer. This is supplemented with risk stratification within these families on an individual basis. This contrasts with secondary screening which is the attempt to discover evidence of the event (e.g. cancer) in those high risk individuals identified by the primary screening process.

Primary screening involves the collection of large amounts of familial and personal data. It permits research into phenotype of pancreatic cancer within and between the different cancer syndromes and within and between the different HP mutation groups. The collection of DNA from both those at risk and controls permits research into identifying the causative genes both for FPC and for further HP genes.

Primary screening is a time consuming undertaking and is normally only formally undertaken by large registries. Some of the registries working in the field of pancreatic disease have already been mentioned in previous sections of the introduction, but they will now be discussed together in the following section.

1.5.1 EUROPAC and Other Registries

The large registries that identify FPC families are administered from three centres in the United States (US) with a further two large registries in Europe. The US centres are the Johns Hopkins University and University of Washington groups and, more recently, the Moffitt Cancer Center in Florida. The National Familial Pancreas Tumor Registry (NFPTR) is the name given to the Johns Hopkins registry. In Europe the two large registries are The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC), coordinated from Liverpool, UK and the German National Case Collection of Familial Pancreatic Cancer or Nationale Fallsammlungt Familiäres Pankreaskarzinom (FaPaCa) of Marburg, Germany.

EUROPAC was established in 1997. It works in close collaboration with both FaPaCa and the French national hereditary pancreatitis registry based in Clichy. EUROPAC is a collaboration of scientists and clinicians that aims to register and treat individuals with inherited pancreatic disease. The groups registered have an increased risk of developing pancreatic cancer giving a rare opportunity for research into the pathogenesis of early pancreatic cancer.

The aims of EUROPAC are: to describe the phenotypes of Hereditary Pancreatitis (HP) and Familial Pancreatic Cancer (FPC), to establish risks to family members of developing these diseases, to identify the gene (or genes) predisposing to the development of pancreatic cancer and to develop methods of screening for early pancreatic cancer in individuals from families considered to be at high risk of developing the disease.

EUROPAC is unique in the volume of personal and environmental data that it collects. Of the North American registries, the one that most closely follows the EUROPAC model is the NFPTR. The University of Washington registry was set up after the incidental recruitment of a family with an unnaturally large number of pancreatic cancers and cases of diabetes mellitus, the so-called 'Family X'[219]. EUROPAC matches all epidemiological and environmental data to DNA from family members wherever possible. If a relevant genetic test is available (see table 1) testing is performed as long as the individual gives informed consent after discussion with a genetic counsellor.

1.5.2 Traditional Risk Stratification: Primary Screening

The familial and personal data are gathered by means of structured questionnaires, as detailed in the methods section of this thesis. At the completion of primary screening the family phenotype or pedigree can be assessed, either by an individual researcher or clinician, or by a multi-disciplinary team. This allows a subjective estimate of the degree of risk within the family to be made.

Estimation of individual cancer risk is more complicated. It is simpler to stratify cancer risk amongst individuals from HP kindreds as the phenotypes (including cancer risk) have been well characterised [91-93] and a genetic test is available for the most common mutations.

Determining cancer risk on an individual basis in FPC kindreds is more difficult as genetic testing is only available in a minority of cases. It was informed by the work of Klein *et al*[185] where risk amongst first degree relatives was determined to be 4.6-fold, 6.4-fold and 32-fold that of the general population, depending on whether there were 1, 2 or 3 family members affected by pancreatic cancer respectively. This study only issued a calculation of risk for a family as a whole. Assuming autosomal dominant inheritance, as previously described, risk is either very high or equivalent to that of the general population, depending on whether an individual is a gene mutation carrier or not. Determining cancer risk on an individual basis within FPC kindreds had never been done before the start of this thesis, and as such, became one of my primary aims.

1.5.3 Current Adjuncts to Risk Stratification

One tool that is currently being used within EUROPAC's trial of secondary screening is the analysis of pancreatic juice gathered at ERCP for cancer associated mutations. The methodologies were published in 2005[220] and have slowly been refined by Dr Yan since then. Molecular analysis of pancreatic juice has the advantage that it gives additional information on individual, rather than familial risk. Molecular analysis is currently used to phase the screening investigations performed as part of the EUROPAC study of secondary screening for early pancreatic cancer (see figure 4) and ultimately, if validated, could trigger prophylactic surgery before a mass becomes visible on conventional imaging. The modality will be described further in section 1.7.2.4.

1.6 Novel Modalities of Risk Stratification

To improve on the traditional stratification offered by careful primary screening and possible genetic testing, new ways of identifying those at risk must be considered. The ideal outcome would be to use a combination of family and individual data (supplemented by basic investigations and genetic testing where available) to give a defined calculation of the risk of cancer to an individual within a set time period. This value, once validated, could become a central part of the decision making process before an individual enters a trial of secondary screening. It should improve the cost effectiveness of screening by avoiding the screening of low risk individuals. It could be used to phase investigations and should reduce the overall number of false positives by targeting screening investigations most appropriately. One way of achieving this outcome is mathematical modelling, possibly with the addition of serum glucose data. Both of these novel modalities will now be examined in turn.

1.6.1 Mathematical and Statistical Models in Pancreatic Cancer

Increasing numbers of mathematical models are being produced to try to stratify or predict risk and guide management in many areas of medicine and surgery. These range from calculations of risk of a myocardial infarction[221], to help guide general practitioners how aggressively risk factors should be treated, to tools such as POSSUM scoring to predict peri-operative death following colorectal bowel resection[222]. Nothing similar has ever been developed in familial pancreatic disease although a model has been produced in Breast-Ovarian syndrome[223].

The volume of EUROPAC's personal, familial and genetic data puts it in a unique position to develop a mathematical model for stratification of risk in high risk groups. Similar models have been developed in other cancers where sufficient volumes of accurate data have been available[224]. Ideally a mathematical model gives a prediction of cancer risk over a fixed time period and needs to be sufficiently user friendly that it can be completed online, (with a calculated level of cancer risk derived from the entered data), within the time frame of a typical clinical consultation.

1.6.2 Serum Blood Glucose

Many studies have shown diabetes to be a risk factor for idiopathic pancreatic cancer. Both retrospective case-control[225, 226] and prospective cohort studies [227, 228] have shown a link, although other studies[229, 230], which excluded those with a short latency period between diagnosis of diabetes and pancreatic cancer, have shown the risk to be more moderate. Overall, two meta-analyses of 20[231] and 36[232] studies have shown a relative risk of 2.0 and a combined summary odds ratio of 1.82 (1.66-1.89) respectively. Not all studies, however, have shown a relationship[229] with one even suggesting diabetes to be protective[230].

In the high risk groups, there is also some evidence of an increased level of risk incurred by diabetes as set out in section 1.4.1 of this thesis. The as yet unpublished EUROPAC research shows diabetes to be an independent risk factor for pancreatic cancer, with a hazard ratio of 2.9 (95% confidence intervals of 1.47, 5.85) in HP kindreds. There is less evidence to prove that diabetes is either a risk factor or a symptom of pancreatic cancer in FPC kindreds, but 'Family X'[219] does show a very high number of cases of diabetes and diabetes status was used as a surrogate marker of carrier status in the *Palladin* paper[113].

There is strong evidence that hyperglycaemia can be a symptom of pancreatic cancer. Up to 80% of those diagnosed with pancreatic cancer have glucose intolerance[233-235] with 40% formally meeting the criteria for diagnosis of diabetes[236]. A greater proportion of new onset diabetics subsequently developed pancreatic cancer up to three years prior to their eventual diagnosis. It has also been shown

that hyperglycaemia often regresses after surgery[132, 234]. The reason or reasons for this remain incompletely understood, but it could be that pancreatic cancer associated diabetes is the result of secretion of diabetogenic peptides by the tumour, with S100A8 having been proposed as a possible agent[131].

Damiano[237] imaged 115 patients aged >50 years over ten years that were admitted with new onset diabetes. Six of the 115 (5.2%) were shown to have adenocarcinoma of the pancreas. A further four patients had either a benign pancreatic tumour or other malignancies involving the pancreato-biliary system, with a further three incidentally detected cancers. The data indicated that new onset diabetes necessitating admission is likely to indicate advanced pancreatic cancer. Of the six pancreatic adenocarcinomas found by Damiano[237], only one was classed as 'early'.

As part of this thesis serum random glucose levels were collected from those with sporadic pancreatic cancer and fasting levels were collected in high risk individuals. Initial results will be shown in chapter 3 of this thesis.

1.7 Secondary Screening

The identification of high risk families raises an ethical dilemma as to how individuals from these families should be managed. This is exacerbated by the low sensitivity of the imaging modalities, meaning that screening could identify as many, or even more, false positives as actual cancers.

Consensus recommendations for secondary screening of high risk groups were proposed at the Fourth International Symposium on Inherited Diseases of the Pancreas[238]. It was concluded that secondary screening should only be carried out on a research basis and only in patients with hereditary pancreatitis, individuals from Peutz-Jeghers kindreds or families with a history of pancreatic cancer. In the latter case the family history should include at least two first degree relatives (or three more distant relatives) unless the participant requesting screening has a mutation in either of the *BRCA* genes or *CDKN2A* (*p16*).

1.7.1 The Rationale Behind Secondary Screening

Successful screening will depend on adequate risk stratification both on a familial and individual level, the advantages of which have been discussed in the previous section. There is already evidence to show that screening for early pancreatic cancer will not be successful if this primary screening is not performed[239, 240]. Serum tests have already been used in both symptomatic and asymptomatic populations and been shown to be ineffective due to poor positive predictive values[239, 240].

The aim of secondary screening of members of high risk groups is to identify pancreatic cancers at a sufficiently early stage in their development that treatment produces an increase in the five year survival rate. This aim will obviously require sufficient numbers of early pancreatic cancers to be detected and treated before a retrospective analysis of prospectively gathered data can be performed. The need for five year survival data, by definition, means that this aim is beyond the scope of a single MD project and will take many years to complete. Secondary aims or additional benefits of detecting and resecting earlier cancers are: firstly, an improvement in our understanding of the development of pancreatic adenocarcinoma; secondly, the elucidation of the stepwise genetic micro-cellular changes that occur as pancreatic cancers develop; thirdly, the proving, refining or rejection of the PanIN model of pancreatic cancer development; and finally, the identification of the ideal time for surgical intervention to maximise chances of survival.

Screening for early pancreatic cancer is only happening on any significant scale within trials organised by a few centres using high risk individuals identified by the registries.

High risk individuals are used, as lives saved by screening must be balanced against the inevitable false positives of screening. Logically, a false positive result of screening for pancreatic cancer must prompt the offer of resection. The mortality of major pancreatic surgery is in the region of 4%, even at large centres[241]. There is also associated morbidity, including endocrine and exocrine pancreatic failure. One study showed a deterioration in glycaemic control in 41% and steatorrhea in 58.6% of all those having Whipples procedure (n=80)[242].

The incidence of pancreatic cancer is below 10 in 100,000 in the general population[20, 243], so if 100,000 individuals were screened with a modality that had 98% specificity there would be 2,000 false positives and only 10 possible lives saved by early detection (even assuming 100% sensitivity). The pre-test incidence of cancer would have to be at least 2% in order for a screening programme with this level of specificity to potentially benefit more people than it harmed.

Only the high risk groups described above offer the possibility of this level of incidence within a reasonable screening window. For example, a 50 year old individual in an FPC family would have a 5% chance of developing pancreatic cancer within a 5 year period based on a 120-fold constant increase in risk over the SEER population[96] (see figure 14a and 14b).

The changes associated with HP detrimentally affect the sensitivity and particularly the specificity of blood testing and imaging, making false positives more likely[244]. However, in other ways, individuals affected by HP are an attractive population for a secondary screening study. The risks of surgery are ameliorated as any resection would be of diseased pancreatic tissue, with affected individuals likely to already have endocrine and exocrine pancreatic failure. Individuals with chronic pancreatitis are also likely to be at a lower risk of an acute episode of pancreatitis following endoscopic retrograde cholangiopancreatography (ERCP) than patients with a normal pancreas[245], making them more suitable for investigation by ERCP than members of FPC kindreds.

Screening also offers important potential research benefits. The early pathogenesis of pancreatic cancer is poorly understood as early tumours are so rarely found. As more small tumours are resected, tissue analysis should lead to the elucidation of the stepwise intracellular changes that take place as tumours develop[246]. The screened population also provide a bank of clinical samples, including blood, urine, saliva and possibly pancreatic juice. These samples can be used retrospectively to test novel molecular screening modalities.

There is limited evidence to guide the age at which screening should commence. The incidence of pancreatic cancer in individuals with HP increases exponentially with age and is negligible before the age of 40[92]. Older patients are more likely to have a cancer at the time of screening, but a younger patient may have more to gain from successful treatment.

In FPC kindreds, the age dependent risk is heavily dependent on family structure. Later generations tend to have an earlier onset of cancer[101], thus maximum risk is at approximately the age of onset in affected siblings and is somewhat lower than the age of onset in affected parents. As the disease mutation is not known, in most FPC families, risk reduces after the age of onset in affected siblings or parents because the probability of being a carrier decreases.

There is little point in screening for early pancreatic cancers, unless there is at least the potential to cure the tumours detected. Two studies from Japan[247, 248] have shown that small cancers (<1cm diameter, no lymph nodes involved), result in a 50-100% 5-year survival rate following resection. These data are inevitably limited by low numbers due to the low incidence of early tumours. There is also some less positive evidence[246] with a retrospective South Korean study showing a 5 year survival of just 23.3% in the 11 stage 1a pancreatic cancers resected as part of their series of 542 cases. Small pancreatic cancers do not necessarily equate to 'early' cancers, but overall these studies show that there is at least the hope of surgical cure when the screening studies detect a malignant lesion.

Once the modalities and protocols have been tested and pancreatic cancer is better understood, screening may, in time, become justifiable in lower risk groups such as new onset diabetics[82] or those with chronic pancreatitis[134].

1.7.2 The Potential Screening Modalities

There is no single screening tool that offers 100% specificity and sensitivity for the detection of early pancreatic cancer. Combinations of modalities may offer adequate positive and negative predictive values in high risk patients, although this has yet to be proven. The five pilot screening programs that are in progress (Johns Hopkins, Washington, Moffitt, EUROPAC and FaPaCa), all use endoscopic ultrasound (EUS) with most centres supplementing this with serum bloods tests. The blood tests will be discussed first, followed by the other potential modalities.

1.7.2.1 Blood Tests

Ideally any screen should be safe and non-invasive. In practice the closest that is possible to this ideal, is a serum test. The two collected by EUROPAC are detailed below.

CA19-9

At least four of the five pilot studies measure CA19-9, a sialylated Lewis antigen produced by patients with digestive tract cancers, particularly those of the pancreas and biliary tree. There is, however, limited evidence supporting its use. Estimates of sensitivity in the literature range from 67-92%, with specificity ranging from 68-92%[249-252]. These values were all obtained using samples from symptomatic patients; when used as a screening modality these figures would be far worse. CA19-9 has never been shown to be effective in detection of tumours in asymptomatic individuals{Kim, 2004 #1829}. Only 50% of cancers less than 2 cm are

associated with a rise in CA19-9[253] and it is rarely elevated in the presence of dysplasia[254]. In a study of 71,000 patients described as asymptomatic undergoing trans-abdominal ultrasonography, CA 19-9 was found to have a positive predictive value of less than 1%[240].

Other Tumour Markers

Other serum tumour markers such as carcinoembryonic antigen (CEA), DU-PAN-2, CA 50, SLX (sialyl difucosyl Le^x), ST-439 (sialyl Le^x-Tn) and CA125 could all be used but have the same drawbacks as CA19-9[255].

Fasting Serum Blood Glucose

The interest in glucose as a possible marker for an emerging pancreatic cancer outlined earlier in this chapter has prompted EUROPAC to gather fasting glucose data in high risk individuals as an additional trial modality. It is unclear whether glucose levels collected within a screening study will be shown to be able to detect potentially curable pancreatic cancers or have a role in risk stratification. In time the results may inform clinical decisions based on imaging, with retrospective analysis of results clarifying their utility. Work continues to develop new serum markers for (early) pancreatic cancer, but there are currently no serum tests that can detect pancreatic cancer at a potentially curable stage.

1.7.2.2 Imaging

Endoluminal Ultrasound (EUS)

EUS is low risk and has a very high sensitivity (>90%) for the detection of pancreatic masses, even in patients with very early tumours[256-258]. It is employed as the primary imaging modality in all the large trials of secondary screening in high risk groups. It has been claimed that it can even detect parenchymal heterogeneity caused by PanIN lesions[259] and that intraductal papillary mucinous neoplasms (IPMNs) can be visualised as cystic masses[50]. EUS therefore meets many of the criteria as the ideal imaging modality in screening, but it does have limitations. EUS is not good at distinguishing between benign lesions and cancers. In a small study (n=85) aimed at distinguishing between chronic pancreatitis and pancreatic cancer, positive predictive value was only 60% based on imaging alone[260]. To improve specificity, EUS has been used to guide fine needle aspiration (FNA) or 'Tru-cut' biopsy from pancreatic lesions. Although, this may still have limited specificity in the presence of abnormal parenchyma[261].

Computed Tomography (CT)

Computed Tomography (CT) produces a three dimensional image of the pancreas using a computer to convert information obtained using conventional Roentgen principles. Interpretation of images is often aided by the use of intravenous and/or gastrointestinal tract contrast. Diagnostic accuracy for CT has been calculated to be as high as 85-90%[262]. A retrospective study showed that abnormalities were

detectable up to 18 months before formal diagnosis with pancreatic cancer[263], but, by definition, these abnormalities can be missed in routine clinical practice. Sensitivity for detecting early cancers is reasonable, but not ideal, with studies having shown sensitivity to be between 69 and 83% and specificity to be between 59 and 93%[264-266]. The problem becomes more acute in the presence of chronic pancreatitis and CT has insufficient resolution to detect PanIN lesions. Tumours below 1cm have been shown to be almost impossible to detect[267]. Clearly this depends on the evaluation of the scans. As the threshold for defining abnormal scans decreases sensitivity will increase but specificity will fall. CT has the further disadvantage that each scan carries a dose of approximately 10 millisieverts (mSv) of radiation for each abdominal CT performed[268]. With some FPC kindreds shown to have a DNA repair defect (*BRCA2*)[108], the repeated use of ionising radiation to image the pancreas needs to be considered carefully.

1.7.2.3 Other Potential Screening Modalities

Trans-abdominal Ultrasound

A variety of other imaging modalities are used by the screening groups alongside EUS. The simplest imaging modality available is trans-abdominal ultrasound (TUS). It is non-invasive, readily acceptable and involves no ionising radiation, but the physical distance from the abdominal wall to the pancreas and the number of tissue interfaces involved requires the use of low frequencies, limiting the picture quality. Whilst the sensitivity of trans-abdominal ultrasound in the detection of pancreatic

cancer is 95% in tumours >3 cm, it reduces dramatically with smaller tumours [269, 270]. Nevertheless, the advantages of TUS mean that it has already been assessed as a screening modality. Periodic TUS checks were performed by Tanaka *et al*[271] in a group of high-risk patients. Patients over 35 years old were recruited on the basis of pancreatic duct dilatation, pancreatic cysts and common bile duct dilatation. Serum amylase, elastase-I, alkaline phosphatase, bilirubin, fasting glucose, CA19-9, CEA and a pancreas-specific TUS were carried out every three or six months. Any abnormality prompted a CT or ERCP with pancreatic juice collection. Of the 393 patients enrolled, pancreatic cancer was diagnosed in 41 patients. Eighteen patients had a surgical resection, three of which turned out to be false positives. Despite these encouraging figures, screening was not necessarily of benefit to these patients. Only four patients had stage I disease at diagnosis and one of these died within three years despite treatment[271].

Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) is fast and non-invasive and produces a three dimensional image of the anatomy of the pancreas without exposing the individual to ionising radiation. The reported sensitivity of MRI ranges from 83-87% and specificity from 81-100%{Muller, 1999 #902;Vellet, 1992 #1802;Muller, 1994 #852}. It has even been reported that T_1 weighted spin-echo MRI can be superior to spiral CT imaging for detection of small lesions[257, 274], particularly when combined with the contrast agent mangafodipir trisodium, which enhances normal pancreatic parenchyma but not neoplasms[257, 275]. Despite these considerable advantages, low resolution and

movement artefacts have previously limited its use[267], although this may change over the next ten years as more modern studies are published.

Magnetic Resonance Cholangio-Pancreatography (MRCP)

Magnetic Resonance Cholangiopancreatography (MRCP) is a non-invasive method of imaging the biliary tree and avoids the risks associated with ERCP. In a prospective study of MRCP using 124 patients referred with a suspicion of malignancy (37 of whom went on to develop pancreatic cancer), Adamek *et al* showed sensitivity to be 84% and specificity to be 94%[276]. Some studies have stressed the value of secretin administration in improving pancreatic ductal details in MRCP[277], but whilst MRCP is a useful, non-invasive tool in the diagnosis of pancreato-biliary obstruction, it has not been fully evaluated in the context of secondary screening. The limited sensitivity even with symptomatic tumours suggests it has limited use as a modality in this context.

Endoscopic Retrograde Cholangio-Pancreatography (ERCP)

Endoscopic retrograde cholangiopancreatography (ERCP) has traditionally been used as both a diagnostic and therapeutic modality in advanced pancreatic cancer with the potential to both obtain cytology and place stents. When used as an imaging modality to identify early pancreatic cancers, its use is less clear cut. It has been used for imaging in both the US secondary screening studies with the emphasis being on the identification of irregular or ectatic ducts with sacculations, which are said to be associated with PanINs[259]. These changes normally occur in the side

branches or in the tail of the pancreas and require an expert radiologist to perform and interpret.

EUROPAC does not use ERCP for imaging but does use it to gather pancreatic juice for molecular analysis for cancer related genetic mutations[220]. Pancreatic juice is the secretion most intimately in contact with tumours and so may contain either tumour cells sloughed from the duct or cell components, including deoxyribonucleic acid (DNA), from necrotic cancer cells. This approach is only suitable for selected patients on a research basis as the potential benefits must be weighed against the risk of inducing acute pancreatitis[278, 279].

During my period of research, administration of diclofenac was added as prophylaxis against post-ERCP pancreatitis. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) commonly used as an analgesic for patients with arthritis or other musculoskeletal pain. Its mechanism of action is incompletely understood but it inhibits prostaglandin synthesis by inhibition of cyclooxygenase (COX). Evidence of its efficacy for prophylaxis of post-ERCP pancreatitis is mixed[280, 281].

EUROPAC are now investigating the potential of performing molecular analysis of duodenal juice gathered after the administration of secretin to eliminate the risk of post-ERCP pancreatitis. This is beyond the scope of this project and no results relating to this will be presented.

Positron Emission Tomography (PET) and CT PET

Positron emission tomography (PET) is a non-invasive method of characterising tissue by measuring the higher glycolytic rate of malignant compared to normal cells. The only PET agent where there is any volume of literature that has assessed clinical utility in pancreatic cancer is 18F-Fluorodeoxyglucose (FDG). Other radiotracers are available but the evidence base is very limited[282].

A meta-analysis (n=387) has calculated the weighted average sensitivity and specificity of FDG-PET to be 94% and 90% respectively. This compares to 82% and 75% for CT[283]. More modern work has, however, given conflicting results, showing FDG-PET to have lower sensitivity than CT[284].

The main strength of PET is its quantitative nature. There is the potential to improve diagnostic accuracy with early work on quantifying tracer uptake[285] or delayed imaging apparently showing malignant lesions to have greater retention of FDG than benign lesions[286].

Despite the attractions of PET imaging there are some serious weaknesses, which are particularly relevant to screening for early pancreatic cancer. Firstly, sensitivity is low for small tumours, probably due to partial volume averaging of signals, although this can be partly addressed by combining PET with CT imaging.

Of particular relevance to the HP group, there is frequent uptake of FDG by inflammatory tissue[287], meaning that PET is unlikely to be a useful screening test in

this subgroup. The same authors also showed that C-reactive protein levels greater than 4 mg/l, could reduce the specificity of FDG-PET to 50%[287].

As discussed in this thesis, hyperglycaemia is frequently seen with pancreatic adenocarcinoma. High serum glucose levels are thought to compete with FDG for glucose transporter sites, reducing the sensitivity of FDG-PET in detecting malignant lesions. Zimny *et al* showed that the sensitivity of FDG-PET decreased from 98% in euglycaemic patients to 63% in hyperglycaemic patients[288].

In summary, these weaknesses mean there is little evidence at present to support the use of PET or indeed CT PET as a screening test for early pancreatic cancer. Further research is required to prove the utility of PET and the specific indications for its use in the imaging of pancreatic disease.

1.7.2.4 Molecular Analysis

No single serum or imaging test is sufficiently sensitive and specific to be used in isolation for screening. The combination of investigations may improve the sensitivity and specificity of the overall process, but any method of sub-stratifying risk within high risk groups should be considered. One potential method is the testing for the presence of cancer related nucleic acid or protein changes in pancreatic juice of high risk individuals. The presence or absence of these cancer related nucleic acid or protein changes can be subjected to a Bayesian analysis to further stratify risk. This has the potential to indicate which individuals have an increased pre-test incidence

and require regular imaging and which individuals can safely have the interval between screening investigations increased.

Modalities for molecular analysis of pancreatic juice have evolved since the early experiments showing that *K-RAS2* mutations can be detected in cellular material obtained at ERCP[289]. *K-RAS2* mutations are almost ubiquitous in pancreatic cancer[290], but unfortunately it was soon established that *K-RAS2* mutations are also common in the pancreatic juice of control patients[291]. Technical difficulties have restricted detection of *Tp53* mutations as a modality for screening, despite a high proportion of *Tp53* mutations in pancreatic tumours and an apparent high specificity for cancer[292]. Various other markers have been investigated including telomerase expression and methylation of specific promoter sequences. Most of these have shown promise, but this has not been sufficient to justify their inclusion as independent screening modalities[293].

EUROPAC has proposed a combination of different molecular tests to phase their screening programme[220]. Cell free pancreatic juice samples are analysed for presence of *K-RAS2* and *Tp53* mutations and quantification of *CDKN2A* promoter methylation. It was proposed that a combination of results with the three molecular tests could stratify risk between negligible and 90% probability of cancer. Stratification is less marked in patient groups with a background of pancreatitis (approximately 0 to 50%), but molecular analysis may conversely have the most impact in HP patients where the sensitivity and specificity of conventional imaging is limited[260]. The techniques have yet to be proven in a prospective study. As things

stand, there is no definite evidence that the molecular markers seen in the juice of sporadic cancer patients are also seen in patients who develop pancreatic cancer as a result of FPC and the risk of inducing post-ERCP pancreatitis goes against the basic principles of a good screening test. On the other hand, the analysis may significantly improve both the sensitivity and specificity of the screening process and inform the screening interval. This should reduce the number of investigations performed and reduce their associated radiation load, reduce the morbidity and mortality from false positives and reduce the financial costs per cancer detected. Initial results of the molecular analysis will be presented as part of this thesis.

1.7.3 Initial Secondary Screening Results from Other Centres

Of the registries already mentioned, The Washington[254, 294], Johns Hopkins[259, 295, 296] and FaPaCa[297] groups have already published initial results of the work on secondary screening. The Moffitt Cancer Center are in a similar position to EUROPAC, where they have published their methods and protocols[298] but have yet to publish their full results. EUROPAC's protocols have been published more than once[96, 299] but data collection remains at a comparatively early stage. Initial results will, however, be shown in Chapter 3 of this thesis.

1.7.3.1 The Washington Group

The University of Washington group screens at risk individuals in FPC kindreds using EUS to provide baseline information. Screening commences ten years prior to the earliest pancreatic cancer death in each individual family. If no abnormality is detected, the EUS is repeated on a twelve monthly basis. If an abnormality is detected that is not thought to be due to pancreatitis, individuals are offered an ERCP after appropriate counselling. If this fails to show an abnormality, follow up is by EUS in twelve months. If both the initial EUS and the subsequent ERCP are abnormal, individuals are counselled and given the option of continuing with surveillance or obtaining a tissue diagnosis. This is achieved by performing a laparoscopic resection of the pancreatic tail[296].

From a total cohort of 75 patients, 15 had abnormalities on EUS and ERCP, all of whom had surgery (12 total and 3 distal pancreatectomies). The three that had a

distal pancreatectomy remain under surveillance. Histology results revealed PanIN-3 lesions in ten individuals and the remaining five specimens contained PanIN-2. Although no cancers were detected in the resected participants, one individual developed an unresectable pancreatic malignancy whilst under imaging surveillance[296].

1.7.3.2 The Johns Hopkins Group

The Johns Hopkins group aims to identify early pancreatic masses when the lesion is either pre-cancerous or a resectable malignancy. Patients from FPC and other cancer syndromes are screened using baseline EUS and CT with imaging repeated on an annual basis. Their screening cohort included 72 at risk members of FPC kindreds and six affected by Peutz-Jeghers syndrome. Primary imaging was by EUS and was performed in every participant. An abnormal scan prompted EUS guided FNA and 65 accepted the offer of an ERCP after appropriate counselling, which was successful in all but one case. Of the 64 ERCPs where the duct was successfully cannulated, there were five cases of ERCP induced pancreatitis and the Hopkins group have expressed the opinion that the benefits of imaging by ERCP do not justify the pancreatitis risk. Sixty seven participants had a spiral CT scan. From this investigative process, 61 participants were felt to have no significant abnormality, with suspected neoplastic lesions present in 17 cases. Of these, 10 continued with surveillance and seven proceeded to subtotal pancreatectomy. The histology from the resections showed IPMNs and PanIN lesions but no cancers. One participant

had a cyst on CT and developed metastatic pancreatic cancer in the interval between imaging and clinical follow up[295].

1.7.3.3 FaPaCa

Langer *et al* [297] reports on seventy six high risk individuals screened between 2002 and 2007 using annual clinical review, serum blood tests and both MRI and EUS. These modalities were supplemented by MR angiography and MRCP in selected cases. A total of 182 examination visits revealed an abnormality in 28 patients. Most of these were found by EUS (n=25), with a further 12 abnormalities visible on MR imaging. There were seven pancreatic fine needle aspirations for cytology and seven patients had operations, supplemented by intra-operative ultrasound. Of those seven individuals that went to theatre, six had a limited pancreatic resection, with the histology showing serous oligocystic adenomas in three individuals, with PanIN1 lesions; PanIN2 lesions; and a PanIN1 lesion plus a gastric type intraductal papillary mucinous neoplasm (IPMN) in the three remaining individuals. Initially, when lesions were detected, surgery was recommended, but the surgical approach became more conservative as the study continued. At the end of the 5 year period, a further 21 were having visualised lesions monitored, rather than operated. Their overall conclusion was that although PanIN lesions can be detected and resected, the low yield and high psychological and financial burdens mean that general screening, even of high risk individuals, cannot be justified at present and should remain limited to the trial setting.

1.7.3.4 The Moffitt Cancer Centre

Klapman *et al*[298] published a paper in 2008 where they outlined their screening protocol but gave no numbers or results. Their approach is based around annual EUS, supplemented by FNA (for lesions >5mm) and CT. They do not report a role for any serum investigations and have not reported any detected lesions or surgical intervention to date.

1.7.4 Cost Effectiveness

Risk and benefit cannot only be considered in terms of patient survival and risk of maleficence. Cost implications cannot be ignored. Papers have discussed the cost of cancer screening in HP[264] and Peutz-Jeghers syndrome (PJS)[300]. Screening of hereditary pancreatitis patients has previously been declared as prohibitively expensive, with a calculated cost of $164,285 per pancreatic cancer detected[264].

In PJS, the cost per life saved was estimated at just $50,000, which is economically viable, but only if all other causes of cancer death in PJS could be eliminated. With existing levels of cancer risk in this syndrome, the cost of screening would rise to a prohibitive $297,000. This cost model also assumed use of molecular analysis to phase screening; without this added element, costs would rise even further to $373,000[300].

In FPC the only work on cost effectiveness[301] came to the conclusion that endoscopic screening was cost-effective, with an incremental cost-effectiveness ratio of $16,885/life-year saved, but this was based on assumptions of a 20% prevalence of pancreatic dysplasia and 90% sensitivity of EUS and ERCP. There are some importance differences between the studies in FPC and PJS. The FPC paper was based on a single round of endoscopic investigations, rather than a protocol guided screening study with repeated investigations. Costs were also based on detection of PanIN lesions rather than on detection of cancers. Analysis of the initial results from the screening studies[299] shows that abnormalities requiring surgery are much lower than the 20% in the Rulyak paper[301] and whilst pre-malignant lesions have been

resected, screening has yet to detect its first pancreatic adenocarcinoma at a curable stage. Estimation is complex, but with the use of molecular analysis, costs in FPC could be below $50,000 per life saved[300]. This will only become clear once several cancers have been detected and the results from the pilot screening programmes have matured. Estimates of financial outlay on screening investigations performed as part of the EUROPAC secondary screening study will be shown in the results section of this thesis.

1.8 Aims and Objectives of this Project

1.8.1 Aims

1. To improve risk stratification on an individual basis in both the HP and FPC groups.

2. To pilot a trial of secondary screening in high risk individuals.

1.8.2 Objectives

1a. To further characterise the phenotype associated with *PRSS1* mutations.

1b. To investigate whether serum fasting glucose levels can differentiate between individuals with differing risk profiles.

1c. To develop a computer model capable of stratifying risk in high risk individuals from FPC kindreds.

2a. Obtain the necessary ethical approvals for a full trial of secondary screening.

2b. To develop a multi-centre collaborative screening network.

2c. To continue with effective primary screening to identify high risk individuals that would benefit from inclusion in the trial.

2d. To test and develop the EUROPAC secondary screening protocol.

2 Materials & Methods

The materials and methods set out in this section are those that pertain to this project. In part, this includes work carried out on behalf of the project by service providers or other members of the research team, for example, imaging investigations were performed by relevant clinicians or NHS departments, serum blood samples were processed by NHS departments of biochemistry and the methods relating to the molecular analysis of pancreatic juice were primarily those of Dr Yan of the School of Cancer Studies. Wherever the methods in this section are those of others, it will be clearly stated.

2.1 Primary Screening

EUROPAC's methods have evolved since 1997 in light of scientific progress and new findings. The methods described are those used and developed during my time as the EUROPAC research fellow. In order to complete the primary screening I was assisted in both data collection and entry by Mr Matthew Harcus, the EUROPAC database manager during my time in post. Primary screening in FPC families received ethical approval from the North West Research Ethics Committee (reference 03/8/069) with the Scotland Research Ethics Committee (reference 04/0/010) giving approval for primary screening in HP kindreds.

2.1.1 Recruitment

Potential participants were either referred by a clinician or they self-referred by contacting the office directly, usually after researching pancreatic cancer on the internet after a death in the family. Referrals were invited from any clinician that had access to individuals at high risk of pancreatic cancer or with HP. This was supported by the EUROPAC study group publishing in peer reviewed journals, by poster presentations at relevant academic meetings and by maintaining close contact with collaborators. Irrespective of the manner of initial contact, after an initial discussion, a patient information sheet (PIS) and questionnaires were sent out for individuals to look through. If they wished to register, they had to provide written informed consent. Consent forms were supplied at the request of the potential participant. The written documentation was supported by either a face to face meeting at the Royal Liverpool University Hospital or collaborating site, or a telephone conversation with the consent form returned by post. One question on the consent form asked for permission to inform the participant's general practitioner (GP) that they had joined the registry. If this consent was given, then a standard letter was sent out for the GPs information and records. Personal, epidemiological, medical and family data were collected by a series of questionnaires. Blood samples were collected from all consenting individuals and stored under the care of the Mersey Regional Genetics Service, based at the Liverpool Women's Hospital. Patients were not recruited when there was any doubt as to their ability to give informed consent. Individuals self-referring to EUROPAC from North America were given contact details for US registries.

2.1.1.1 Genetic Testing

DNA was collected and stored in compliance with the Human Tissue Act 2004 (England and Wales), which was amended in 2006. This was under the care of the Mersey Regional Genetics Service, where all testing took place. In FPC families, if the history suggested the presence of a particular testable mutation (e.g. in the *BRCA2*, *CDKN2A, or STK11* gene), testing was offered after the individual had been referred to a clinical geneticist for a discussion about the potential implications of a positive result. Genetic testing in HP families, where the history suggested a highly penetrant mutation, started with testing for the common mutations in the *PRSS1* gene. If the common mutations were not found, the entire gene was sequenced. A proportion of HP families with a phenotype suggesting a highly penetrant mutation were shown to have a normal *PRSS1* gene. EUROPAC classified these families as 'Neg All HP'. If *PRSS1* testing was negative or the phenotype suggested a low penetrance mutation, individuals were tested for the 33 most common disease related Cystic Fibrosis Transmembrane Receptor (*CFTR*) mutations, as well as the p.N34S variant of *SPINK1*.

2.1.2 Data Storage

All collected data were stored using Progeny software (version 7.01) on a password protected computer isolated from the internet. Data were stored in compliance with the Data Protection Act (2003). The ethical agreement permits a single copy of the database to be kept in a locked fireproof box in a second locked office. Access to the database was limited to the EUROPAC research fellow, the database manager and Dr W Greenhalf, the lead scientist on the project.

2.1.3 Outcome of Primary Screening

On completion of the initial process of primary screening, the Progeny software was used to construct a family tree summarising the phenotype and there was a multi-disciplinary discussion by a team of clinicians, scientists, and where possible, a clinical geneticist. The purpose of the discussion was to use all available data to classify the kindred or suggest any further work that could be performed before the family was formally accepted onto the registry. In most cases I was one of the clinical representatives.

The diagnosis of FPC in a family is based on evidence of autosomal dominant inheritance of predisposition specifically for pancreatic cancer. Recruitment required at least two first-degree, or at least three second-degree, relatives in two or more generations with confirmed pancreatic ductal adenocarcinoma. Confirmation of cases was by: histological evidence, cancer registry confirmation, or good quality medical notes with death certificate evidence, in that order. The real purpose of the multi-disciplinary discussion outlined in the previous paragraph was to pick out

kindreds where there were two or more cases of pancreatic cancer, but the causative genetic syndrome was something other than FPC. Some families with only one pancreatic cancer death were enrolled in the presence of a proven genetic mutation associated with an increased risk of pancreatic cancer (e.g. *CDKN2A*). Where a mutation was identified, families were classified according to the underlying cancer syndrome, irrespective of the number of pancreatic cancer deaths in the kindred.

Similarly, the classification of HP was based on autosomal dominant inheritance, in this case of pancreatitis. Recruitment required evidence of acute or chronic pancreatitis in at least two first-degree, or at least three second-degree, relatives in two or more generations. Diagnosis of chronic pancreatitis was made by: histological evidence, a low faecal elastase or evidence of pancreatic calcification on imaging, or on the basis of good quality medical notes, in that order. Classification was easier in HP kindreds as it was normally supported by the results of genetic testing and kindreds were largely classified by *PRSS1* mutation. Families were enrolled as 'Neg All HP' if their phenotype indicated autosomal dominant aetiology and no genetic cause was identified after sequencing of the *PRSS1* gene. Some individuals were recruited with a phenotype that suggested sporadic disease if genetic testing results indicated the presence of a chronic pancreatitis associated mutation such as the p.N34S mutation of *SPINK1*. Attempts were then made to register other family members. Individuals with apparent sporadic idiopathic pancreatitis were not registered in the absence of a proven genetic mutation.

The FPC arm of the registry was divided into sub-groups. Pancreatic cancer families were grouped by phenotype as 'FPC', '?FPC', 'With Gastric', '*BRCA2* FPC', and 'Other'. The true 'FPC' group contained families where the phenotype was definitely consistent with autosomal dominance, with multiple cases of pancreatic cancer in multiple generations, and the cancer cases had been proven. The group '?FPC' contained families with multiple pancreatic cancers but the strict criteria laid down to be classified as 'FPC' were not met. This could be for a number of reasons, but in practice was most commonly because the cancer cases were in a single generation (suggesting a multigene or environmental cause) or sufficient numbers of cancer cases could not be proven. 'With Gastric' was the name given to a group of families that fully met the criteria for diagnosis as 'FPC', but also contained an additional case of gastric carcinoma. The cases could not be explained by any of the known genetic syndromes and the cancers in these kindreds may be the result of an as yet unidentified mutation, different to that causing the cancers in the FPC kindreds. At present, the data remain too few to investigate this further. *BRCA2* FPC contained families where the phenotype was consistent with 'FPC' in the presence of a BRCA2 mutation. These families did not have multiple cases of breast or ovarian cancer, or they would have been classified as being 'Breast Ovarian' (see below). One '*BRCA2* FPC' family had a single case of ovarian cancer, but this individual proved not to be a BRCA2 mutation carrier. The 'Other' group contained any families registered that did not meet the criteria for any of the above groups. This group contained the rare cancer related syndromes, for example, FAMMM-PC and Breast-Ovarian families that contained a pancreatic cancer. Families with single cases of pancreatic cancer

were not registered unless a genetic mutation associated with pancreatic cancer had been identified. Basic data relating to these classifications are set out in table 3 of the results section of this thesis.

The HP arm of the registry was also sub-divided. The sub-groups for the pancreatitis families were largely based around the results of genetic testing. True HP kindreds were classed as belonging to the 'p.R122H', 'p.N29I', 'p.A16V', or the 'Neg All HP' groups. As stated above 'Neg All HP' was the name given to kindreds with a phenotype entirely consistent with HP but no cause had been identified in the *PRSS1, CFTR* or *SPINK1* genes. Individuals with the p.N29T mutation were classified as belonging to the p.N29I group. At time of data guillotine for the primary screening results displayed in this thesis, individuals with a p.R122C mutation were included in a group entitled 'Other' *mutation.* This contained HP families with proven rarer *PRSS1* mutations, for example p.R116C, or p.V39A. The reasoning for p.N29T being included with p.N29I is that biochemically the substitution of an isoleucine or a threonine is similar (both are small neutral amino acids). In contrast cysteine (given its propensity for formation of sulphur bridges) could result in very different activity. Simon *et al*[302] have shown that p.R122C results in a very radical change in both autoactivation and autodegradation, which is far more dramatic than p.R122H. Two final groups on the HP database were 'HP family' and 'HP problem'. The group 'HP family' contained kindreds where the phenotype was consistent with HP and genetic testing should have been possible but had not been initiated or was in process. 'HP problem' was where the proband wanted to be on the registry, but had declined

genetic testing, normally after discussion with a genetic counsellor. Basic data on these classifications are set out in table 4 in the results of this thesis.

There were also a number of sub-groups that were relevant to non-HP kindreds. These were 'FIP', 'Sporadic', 'IPCA' and 'CFPANC'. 'Familial Idiopathic Pancreatitis' (FIP) was where there were several cases of pancreatitis in a family but these were restricted to a single generation or were in family members where the relationships are sufficiently distant that the cases were not in keeping with HP. 'Sporadic' was a single idiopathic case and included those with *SPINK1* mutations. Families where there was one case of idiopathic pancreatitis with an additional pancreatic cancer case (IPCA) is self-explanatory. These families may or may not have a proven *SPINK1* mutation. The final group 'CFPANC' (*CFTR*-related idiopathic pancreatitis) had one or more cases of pancreatitis in individuals with a proven *CFTR* mutation. Basic data on these classifications are set out in table 6 of the results section of this thesis.

Classification in both the FPC and HP arms of the registry was an ongoing process and in some cases the group in which a family had been placed changed as additional family members were recruited, further genetic testing was performed, or additional cancers developed or were proven. As consent was required before primary screening could take place, some families were subsequently shown to have neither FPC nor HP. During my time in post, the data on these families were kept in a 'pending' file and a file number was not allocated until they were formally classified.

Informed pedigree analysis at time of classification identified the high risk individuals within kindreds. Efforts were then made to see whether these individuals were interested in registration via previously recruited family members, the process was repeated and the kindreds were characterised as fully as possible.

2.2 Risk Stratification

2.2.1 Manual Methods of Risk Stratification

During the course of this work the methods employed for risk stratification improved in accordance with the aims of the thesis. However, recruitment of participants for screening was ongoing throughout this period using less sophisticated approaches. The manual method of risk stratification was the result of analysis of the phenotypes built up by the thorough primary screening described in the preceding section. Informed judgement was used to identify those family members at particular risk. For FPC families this was a subjective calculation dependent on the number of confirmed pancreatic cancer in the family, the likelihood that an individual was a carrier of an apparent predisposing genetic mutation, individual risk factors and genetic testing where available. In HP kindreds, there was a similar process but ultimately the final decision for each individual was less subjective because a diagnostic genetic test was available for all but the 'Neg All HP' kindreds and the cancer risk in HP is well characterised, at least in p.R122H and p.N29I kindreds[91-93].

2.2.2 Novel Modalities

2.2.2.1 Glucose

Serum glucose samples were collected from consenting asymptomatic members of FPC kindreds, with additional samples collected from high risk individuals (from both FPC and HP kindreds) as part of the secondary screening study. These were fasting samples, normally taken in a primary care setting and processed locally with the result sent by post or fax. Some samples were collected in the outpatient setting. Samples obtained from participants in the Liverpool area or from patients who were treated at the Royal Liverpool University Hospital were processed using NHS facilities at the hospital. There was obviously some variability in how fasting glucose samples were processed in different institutions depending on local infrastructure and standard operating procedures. The process used by the biochemistry department at the Royal Liverpool University Hospital is described in section 2.3.3.1 alongside the other investigations used as part of the secondary screening study.

All results, whether processed locally or at collaborating centres were stored on the main EUROPAC Progeny database under the registry ethical agreements for FPC (MREC 03/8/069) and HP (MREC/04/0/010) respectively.

2.2.2.2 Computer Modelling

The desire to improve risk stratification and make this as objective as possible prompted the development of a computer model to stratify risk in FPC kindreds. This was done in collaboration with two members of the University of Liverpool Clinical

Engineering Department (employed by the Royal Liverpool & Broadgreen University Hospital Trust) and a PhD student, Francesco Tortora, who worked alongside them. My role in this process was: primarily commissioning; but I also generated, checked and coded the dataset; acted as the link with the non-clinical members of the team; and tested the interface generated by Mr Tortora to identify any problems so that they could be resolved.

The dataset used to produce the model was formed from 85 families from the FPC database with three or more cancer cases. These kindreds contained 1251 individuals, of whom 297 had been affected by pancreatic cancer. The specific fields used were the individual's age, gender, the age of death of an affected parent and smoking status. Smoking data were only available for 380 individuals. The gender specific probability of smoking was calculated from these individuals for each year of birth. Dummy variables were then generated for smoking in the individuals with no data so as to maintain the same probabilities across the whole group. Together these data made up the primary inputs into the equation shown below in figure 2.

Figure 2: The Equation Used in the Mathematical Model

$$\log t_i \sim N(\mu_i, \tau)$$

$$\mu_i \leftarrow \langle \beta, z_{f_i} \rangle + \beta_0 + b_{f_i}$$

$$b_{f_i} \sim N(0, \eta)$$

$$\beta_j \sim N(0, v_j)$$

$$v_j \eta, \tau \sim G(1/1000, 1/1000)$$

This figure shows the mathematical equation developed using EUROPAC data to predict cancers in FPC families. The expressions on the first line describe the log of time (t_i) being normally distributed, with ($N(\mu_i, \tau)$) being a normal distribution term. Within this term (N) describes the normal distribution, μ_i is the mean and τ is the inverse variance. The second line states that the mean (μ_i) is defined as ($\leftarrow$) the scalar product ($\langle\ \rangle$) of the vector of the model co-efficients (β,) and the vector of the covariates (z_{f_i}) *plus* a fixed term (β_0) *plus* the frailty or family clustering term (b_{f_i}). The co-variates are individual values e.g. gender, smoking, age class for death of parent and the co-efficients are the values by which these covariates are multiplied. Line three describes the frailty term (b_{f_i}) which is again normally distributed ($N(0, \eta)$), with 0 representing the mean and η being the inverse variance. The fourth line describes the elements of the beta vector (β_j) as being normally distributed (N), with 0 again being the mean and v_j being the inverse variance. The fifth line states that nu (v_j), eta (η) and tau (τ) are gamma distributed ($\sim G$), meaning that the variables will always be positive.

Dr's TakTak and Eleuteri both had experience in computer modelling in malignancy[224, 303-305] and used the data to construct an accelerated failure time model. A frailty factor was used to account for familial aggregation[306]. The model was formulated in the Bayesian framework using WinBUGS software[307-309]. The

specific covariates used were individual values for age class, gender, smoking, age class for time of death of an affected parent.

The model output was then multiplied by two factors that increased the model's predictive power. These factors were the 'family index', and the 'probability of being a carrier'. The family index was intended to account for the difference in family size, where some kindreds had a phenotype with three cancer cases out of a total of six on-kindred individuals and other kindreds had three cancers within a large family of perhaps 50 at risk individuals. Each affected individual makes it more likely the family has a genuine increased risk and each unaffected individual makes it less likely. The smaller family logically has a greater likelihood of a genetic cause for the cancer cases. The index was calculated by dividing the number of cancer cases in the family by the number of individuals at risk i.e. on kindred individuals greater than 40 years, giving a result of between zero and one.

The probability of being a mutation carrier was derived manually by looking at the pedigrees, with a value entered for each individual. Assuming autosomal dominant inheritance, any first degree relative of an affected individual, where the cancer had resulted from a genetic mutation, had an even chance (a probability of 0.5) of carrying the responsible mutation themselves. If a first degree relative remained unaffected, then the probability of their offspring being a gene carrier reduced to 0.25.

This equation in figure 2 was tested for its powers of 'discrimination' and 'calibration' and adjusted until it had been optimised. 'Discrimination' is the ability of the model to

separate the subjects into two groups, in this case those that developed cancer and those that did not. It was tested using Harrell's C-Index which is an extension of the Area Under the Receiver Operating Characteristic (AUROC) Curve. Harrell's C-Index is thought to be better for survival analyses as it is threshold independent and can cope with censored data[310, 311]. It is calculated by pairing all samples that are comparable and calculating the probability of their concordance. For any given pair to be *comparable*, at least one of the subjects in the pair must have developed the event (e.g. pancreatic cancer) and the follow-up time period for this subject must be less than that of the second subject. For a pair to be considered *concordant*, the probability of survival predicted by the model for the second (unaffected) subject must be greater than that predicted for the first.

Figure 3: Harrell's C-Index

$$\text{C Index} = \frac{\text{Probability of Concordance}}{\text{Probability of Comparability}}$$

Figure showing Harrell's C Index, which was used to test the mathematical equation for its power of *discrimination* (its ability to differentiate between those that developed cancer from those that did not). Affected and unaffected individuals were paired and a numerical value was calculated by dividing the probability of a pair being *concordant* (the probability of survival predicted by the model for the unaffected subject had to be greater than that of the affected one) by the probability that the pair was *comparable* (at least one of the subjects in the pair must have developed pancreatic cancer and the follow-up time period for this subject had to be less than that of the second subject.)

The second method used to test the model was 'calibration'. This is the degree of correspondence between the probability of an event being predicted by the model and the probability of that event having actually occurred. It is tested using Hosmer-Lemeshow analysis which has previously been peer reviewed as suitable for this type of work[312]. Values derived from the model were first rank ordered and split into five groups, with the most likely to develop pancreatic cancer in group one and the least likely in group five. Predicted versus actual survival were then compared and tested for goodness of fit with the results quantified as a Chi-square statistic.

The Computer Interface

The interface was constructed by Mr Francesco Tortora, a PhD student, who was supervised by Dr's TakTak and Eleuteri. My role in the creation of the interface was to devise an algorithm for how the data were to be collected, which he converted into an online interface using HyperText Markup Language (HTML). I used the interface to enter test data and noted any errors and problems, which Mr Tortora then corrected. At the end of data entry for each high risk individual, a summary of the data was produced. If this summary was accepted as correct by the individual entering the data, a survival curve was produced which gave a calculated estimate of risk from pancreatic cancer for that individual over the next five years.

Pseudo-Prospective Testing

Once the model had been tested and optimised, its ability to predict cancers was tested by backdating the EUROPAC FPC database to the year 2000. Between 2000

and 2008, there had been a total of 27 cancers amongst FPC kindreds. MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium) software was used to perform a Receiver Operating Characteristic (ROC) curve analysis to determine the optimum level of risk at which screening should be triggered. This was set so that all cancers were within the screened group, but unnecessary screening was minimised, as shown in table 8 of the results section of this thesis.

2.2.3 Improving the Characterisation of p.A16V

Mutation type was used in this study as a variable in quantifying cancer risk in HP. Although p.R122H and p.N29I had previously been extensively studied little information was available on p.A16V. Therefore, as part of this work, the phenotype of pancreatitis, pancreatic failure and cancer in p.A16V kindreds were researched. The difficulty of characterising p.A16V at the start of this thesis was that this form of mutation was too rare for single centres to perform a meaningful statistical analysis. Most kindreds used in this study had already been recruited and characterised by EUROPAC with primary screening performed in the standard way. Additional participants were recruited by contacting all prominent researchers in the field of HP identifiable from the literature. I was ably assisted in this by my colleague Mr Matthew Harcus. Data and DNA were obtained wherever permitted using existing local ethical consents. No data were accepted without evidence of written informed consent that permitted transfer or sharing of data. The data obtained were analysed using SAS Institute Inc. StatView version 5.0. Endpoints including onset of pancreatitis and diagnosis of endocrine and exocrine pancreatic failure were analysed using the method of Kaplan-Meier, with differences assessed using the Mantel-Cox logrank test. Where an endpoint was not reached, censor times were taken as the age of last contact. Differences in median values for continuous data were tested using the Kruskall-Wallis (for comparison of multiple groups) and Mann-Whitney-U tests (for comparison of two groups).

2.3 Secondary Screening

2.3.1 Recruitment and Inclusion and Exclusion Criteria

As part of this study I made two ethical applications for a trial of secondary screening, one in the FPC group and a second in HP kindreds. These were titled '*The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) Study of Secondary Screening for Early Pancreatic Cancer in Familial Pancreatic Cancer Kindreds*' granted by Warwickshire Research Ethics Committee (REC Reference 07/H1211/96) and '*The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) Study of Secondary Screening for Early Pancreatic Cancer in Hereditary Pancreatitis*' granted by Central Manchester Research Ethics Committee (REC Reference 07/H1008/153) respectively.

All participants in the EUROPAC secondary screening trial were recruited from the main registry. Participants joining the registry from the time I took up the post as the EUROPAC research fellow were made aware of the secondary screening trial on registration. Those that had joined the registry between 1997 and 2006 were approached by post, providing that they had consented to being contacted about further research opportunities at the time of their recruitment. In practice many of kindreds recruited to the FPC arm of the registry during my time in post contacted the EUROPAC office having searched online for information about pancreatic cancer screening after the diagnosis of a pancreatic cancer in a close family member.

Initial information and the trial documentation were made available either by post or email. If high risk individuals were interested in joining the study, they obtained a referral from their GP to one of the consultant pancreatologists working on the project. In practice, this was almost always Professor Neoptolemos. The individuals were seen in the outpatient clinic, where the limitations and risks of the available modalities were explained. Participants were recruited if: they wanted to go ahead; they met the inclusion and did not meet the exclusion criteria for the respective arm of the trial (see table 2); and they provided written informed consent following a suitable 'cooling off' period.

Table 2: Inclusion and Exclusion Criteria for EUROPAC Studies of Secondary Screening

Table summarising the inclusion and exclusion criteria for the secondary screening study for high risk individuals from both FPC and HP kindreds. In the FPC group, the criteria for FPC had to be fully met i.e. there had to be at least two confirmed pancreatic cancer deaths in at least two generations. The only exception to this was for individuals from kindreds with single cancer cases, if a pancreatic cancer associated mutation had been identified in both the affected individual and the individual wishing to be screened. The usual starting age for screening was 40 years, as the risk was minimal before that point, although this could be adjusted depending on the outcome of primary screening and the ages of other cancer deaths. Exclusions in the FPC group were on the basis of age, females that were either pregnant or had not taken contraceptive measures, those unable to provide informed consent and members of kindreds with a testable pancreatic cancer associated mutation (e.g. BRCA2) that had been tested and shown to be *wild type*. In the HP group, these criteria were identical, but in practice, genetic testing results carried far greater significance.

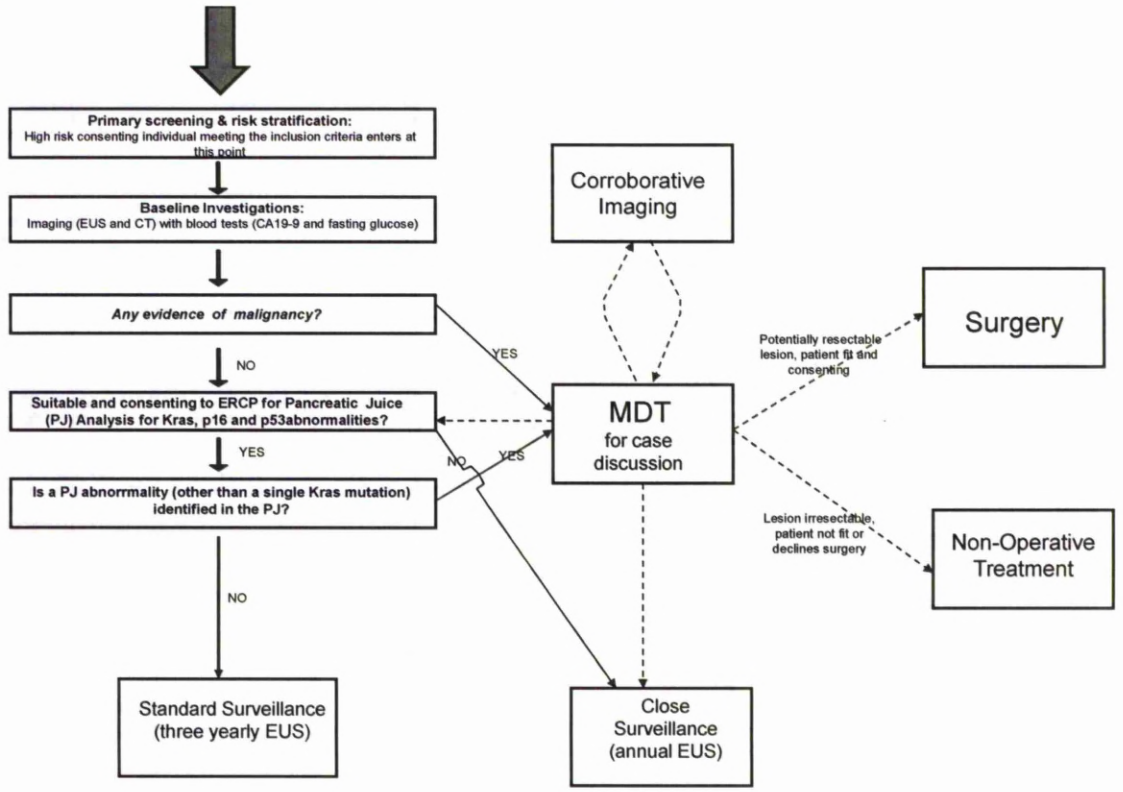
	Inclusion Criteria	Exclusion Criteria
Familial Pancreatic Cancer (FPC)	1. Any patient from a confirmed familial pancreatic cancer kindred over the age of 40 years. There is no maximum age limit to the screening process. The diagnostic criteria for Familial Pancreatic Cancer are: • Two first degree relatives with pancreatic ductal adenocarcinoma; *and/or* • three or more relatives with pancreatic adenocarcinoma; and/or • Individuals from families with a causative gene linked to pancreatic cancer (e.g. *BRCA2*), where the individual has tested positive for the mutation and has a first or second degree relative that has been diagnosed with pancreatic cancer. 2. Individuals aged under the age of 40 years can be included on an individual basis if their phenotype suggests a particularly high risk. This flexibility in individual cases is to account for 'anticipation' and the recruitment decision takes place after multi-disciplinary discussion.	1. Individuals under the age of 40 years with the exception of a small proportion of individuals from pancreatic cancer families with particularly young onset disease. 2. Any patient unable to give informed consent. 3. Any woman able to bear a child but who has not taken appropriate contraceptive measures. 4. Those testing negative for a pancreatic cancer associated mutation, where a genetic test is available.
Hereditary Pancreatitis (HP)	1. Any patient with hereditary pancreatitis over the age of 40 years. There is no maximum age limit to the screening process. The diagnostic criteria for Hereditary Pancreatitis are: • At least two relatives with chronic pancreatitis in at least two generations in the absence of gallstones or a definite correlation with alcohol excess; and/or • Individuals of any family who carry a predisposing mutation for hereditary pancreatitis.	1. Individuals under the age of 40 years with the exception of a very small proportion of patients younger than the age of 40 years with hereditary pancreatitis who will undergo an ERCP as part of their routine clinical management. 2. Any patient unable to give informed consent. 3. Any woman able to bear a child but who has not taken appropriate contraceptive measures. 4. Those testing negative for a detectable pancreatitis causative mutation, where a genetic test is available.

2.3.2 The Secondary Screening Protocol

For those individuals that met the inclusion criteria, wanted to enter the secondary screening study and provided written consent, the next step was the baseline investigations. These included serum blood investigations (fasting glucose and CA19-9) and imaging (EUS and CT). The step following these baseline investigations depended on initial results and the individual's attitude towards which screening investigations they chose to take up. The algorithm for the FPC arm of the study is set out below in figure 4 and it will be this which will be outlined below. The only real difference between the algorithms for FPC and HP was that the imaging modality of choice in HP individuals was normally CT rather than EUS.

Any detected abnormality was discussed in the meetings of the EUROPAC study group and those considered potentially significant were taken to the regional pancreatic cancer multi-disciplinary team (MDT) meeting. If the MDT considered a detected abnormality to represent a potential malignancy, the individual would have been staged in the normal way and the tumour resected if possible, with the individual treated non-operatively in the presence of locally invasive or metastatic disease. The MDT might have decided that further investigations were indicated. This could either have been additional imaging or molecular analysis of pancreatic juice. Once the case had been fully assessed, if no definite diagnosis of cancer had been made, the participant would have remained on the close surveillance pathway with annual investigations.

Figure 4: The EUROPAC Secondary Screening Protocol



The EUROPAC protocol for secondary screening in high risk individuals from FPC kindreds as it was at the end of my period in post as the EUROPAC. The first step is thorough primary screening. Individuals who meet the entry criteria and consent to join the study go forward for baseline investigations. If a relevant abnormality is detected, the results are discussed at the regional pancreatic cancer multi-disciplinary team (MDT) meeting. Their decision options are shown by the arrows with dotted shafts. If there is a definite malignancy present, the participant will be considered for surgery where possible; if the tumour is inoperable or there is already metastatic disease present, the participant will be treated non-operatively. The MDT may decide that further investigations are indicated. This may include additional imaging or molecular analysis of pancreatic juice (PJ). Once the case has been fully assessed, if no definite diagnosis of cancer has been made, the participant will remain on the close surveillance pathway with annual investigations. If baseline investigations are normal and MDT discussion is not indicated, an ERCP for pancreatic juice analysis is offered. Those that have an ERCP and are shown to have either no genetic abnormalities or a single Kras mutation in their PJ enter the standard surveillance pathway, where imaging and PJ analysis are staggered within a three year cycle. Those with multiple mutations or declining ERCP enter the close surveillance cycle of annual imaging.

If the high risk individual had their baseline investigations and did not require referral to the MDT, there was the option to perform an ERCP for juice collection. If informed consent was given and the analysis showed any abnormality other than a single Kras mutation, the case would be discussed at the MDT and the participant would enter the close surveillance pathway of annual imaging. If juice analysis revealed either no genetic abnormalities or a single Kras mutation, the participant entered a standard surveillance pathway with further investigations staggered within a three year cycle, for example, an EUS 18 months later and a further ERCP three years after the first one. The *standard surveillance* pathway therefore meant that one ERCP and one imaging investigation would be performed within a three year cycle. The *close surveillance* pathway meant that one imaging investigation would be performed per year which might or might not be supplemented by one or more ERCPs and/or corroborative imaging, depending on other results.

If baseline imaging revealed normal pancreatic parenchyma, the imaging investigation of choice was EUS. If baseline imaging showed chronic pancreatitis to be present, the imaging investigation of choice became CT. In practice, this meant that the primary method of imaging in the HP group was almost always CT, with EUS almost always being the main imaging modality in the FPC kindreds. Other imaging modalities were used for corroborative imaging as recommended by the MDT.

The clinician and the participant decided what elements of the screening programme were appropriate and acceptable. Participants could opt in or out of each aspect (i.e. bloods, imaging and pancreatic juice analysis). The baseline results and the

participant's attitude to ERCP effectively determined whether the participant entered the *standard* or *close surveillance* pathway as set out in figure 4.

2.3.3 Secondary Screening Investigations

I usually took the blood for the serum sampling but this was processed by NHS staff in the relevant department of the Royal Liverpool University Hospital (RLUH) or at a collaborating site. The imaging was performed in the relevant part of the radiology or gastroenterology department either at the RLUH or at a collaborating site. The EUS and ERCPs were performed by either consultant radiologists or gastroenterologists at either the RLUH or other collaborating centres. The methods outlined below relate to those applied at the RLUH at the time I was co-ordinating the secondary screening study.

2.3.3.1 The Blood Investigations

Technical Aspects of Testing for CA19-9 at RLUH

Blood was collected by venepuncture, packaged in a standard lithium heparin tube and transported to the biochemistry laboratory. The sample was then centrifuged to obtain the 10μl of plasma required for the assay. Plasma samples were stored and processed as a batch twice weekly.

The technique employed, which has been standardised against the Enzymum-Test CA19-9 method, was an automated version of a Sandwich Enzyme Linked Immunosorbent Assay (ELISA) performed using a Roche Modular E170 analyser. There were three phases to the process, the first two were incubations and the final phase was the measurement phase. At the first incubation, 10μl of sample, biotinylated monoclonal CA19-9 specific antibody and a monoclonal CA19-9 specific

antibody labelled with ruthenium complex formed a sandwich. Following the addition of streptavidin coated microparticles, during the second incubation the complex became bound to the solid phase via interaction of streptavidin and biotin. In the measurement phase, the reaction mixture was aspirated into the measuring cell where microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with Procell. Application of a voltage to the electrode then induced chemiluminescent emission which was measured by a photomultiplier. Results were determined by the analyser via an instrument specific calibration curve. This was generated by a 2-point calibration performed on the instrument and a master curve encoded on the reagent bottle barcode. The three phases of the assay took a total of 18 minutes.

The result was expressed as units per litre, or more usually thousands of units per litre (kU/L). The normal range was <35 kU/L. A result could be determined with the standard method described for levels ranging from 0.600-1000 kU/L, with values below the detection limits reported as <1.0 kU/L. Values >1000 kU/L prompted dilution of the sample with Elecsys Universal Diluent at a 1:10 to permit the process to be repeated and a value to be obtained.

Once the result had been calculated, biomedical scientists within the department either released the results onto the *Telepath* computer system, which authorised the result or on occasion the endocrine duty biochemist authorised results manually after clinical validation. The final stage was the reporting of results electronically to other Royal Liverpool and Broadgreen University Hospital Trust (RLBUHT) computer

systems and external systems where appropriate. A printed report was forwarded to the requesting clinician. Results were available within 7 days and, after testing, samples were frozen in a -20°c freezer and stored for three months.

Technical Aspects of Testing for Serum Blood Glucose at RLUH

The biochemistry department processed samples taken at the Royal Liverpool University Hospital. A fasting blood sample was collected by venepuncture and packaged in a fluoride EDTA plasma tube. The tube was transported to the laboratory and plasma samples were centrifuged within an hour of receipt. Analysis was performed using a 'Roche P' automated analyser and was achieved by oxidisation of glucose by glucose-oxidase (GOD) in the presence of atmospheric oxygen to form gluconolactone. Hydrogen peroxide was formed which oxidised 4-aminophenazone and phenol to 4(p-benzoquinone-monoimino)-phenozone in the presence of peroxidase (POD). The process resulted in a red dye, with the colour intensity being directly proportional to the glucose concentration. The value was determined by the analyser using spectrophotometric principles.

The result was expressed as millimols/litre (mmol/l). The detectable range varied from 0.11 to 25 mmol/l. Values under 2.5 mmol/l would have prompted a second test and levels >25.0 mmol/l would have prompted repeat analysis using a reduced sample volume diluted with saline. An error up to 1.8% was included within the results obtained.

Biomedical scientists were responsible for releasing results to the Telepath system. This process auto-validated the result or the result could be authorised manually by the duty biochemist. After authorisation results were reported electronically to other RLUH and external computer systems, with a paper copy being sent to the requesting clinician. Glucose was assayed daily and results were available the same day. Samples were retained in a cold room for one week after testing.

2.3.3.2 The Primary Imaging Investigations

Technical Aspects of Performing EUS at RLUH

Endoluminal ultrasound examinations were conducted at several collaborating centres, but the process at the RLUH will be described. Consenting patients attended the department of gastroenterology, normally as an outpatient. The scan was performed using analgesic throat spray just prior to intubation. Benzodiazepine sedation was given as required but was rarely needed. Participants were intubated with an Olympus GF-UE260 scope. Visual images were obtained of the upper gastrointestinal tract before ultrasound images were obtained of the pancreas using ultrasound frequencies of 5-20 megahertz (MHz). Images could be both stored and printed. A report was entered on the Unisoft system and images were uploaded to Picture Archiving and Communication System (PACS). The participant then underwent a period of observation in the recovery suite before being discharged home.

Technical Aspects of Performing Computed Tomography Scans at RLUH

Computed tomography images were obtained at the Royal Liverpool University Hospital using a Siemens Sensation 16 helical multi-slice Computed Tomography scanner. Consenting patients attended the RLUH department of radiology, normally as an outpatient. The scan was performed following the RLUH enhanced pancreatic protocol. The participant was given one pint of water immediately before the scan to distend the stomach and duodenum, they were positioned on the scanning table in the supine position with their arms outstretched above their head, with nipples and the midline being the centring points. The scan was conducted in two parts, the arterial and venous phases, lasting 40 and 25 seconds respectively. In the arterial phase, 100 mls of intravenous iodinated contrast medium was injected at a rate of 3 mls per second, with slices taken at 1mm increments. The venous phase was essentially a repeat of the arterial phase but the delay between the administration of the contrast and conduction of the scan gave an alternative view of the same structures.

The data obtained from the scan were simultaneously transmitted to a Siemens Leonardo CT workstation in the adjoining room and to the PACS system. The images were available on the RLUH computer system via PACS immediately and these were supplemented by a report once the responsible radiologist with a pancreatic sub-speciality interest had reviewed the scan.

2.3.3.3 Secondary Imaging Investigations

Technical Aspects of Performing USS at RLUH

Trans-abdominal ultrasound scanning of the pancreas was performed at RLUH using a GE Logic 9 scanner with a 4-1 MHz curvilinear transducer. The transducer was placed against the abdominal wall of an individual who normally lay in the supine position. Aquasonic ultrasound gel was used as an interface to maximise image quality. The pancreas was assessed in real time, with images of the pancreas taken in the axial and sagittal sections. The machine's 'TruScan' technology enabled ultrasound data to be both digitally acquired and stored in its raw data format. This enabled the study to be subsequently re-accessed and reviewed, after the patient had left the department. A report was dictated after each procedure and saved images were uploaded to the PACS system.

Technical Aspects of Performing MR and MRCP at RLUH

Magnetic Resonance Imaging (MRI) scans were obtained at the RLUH using a Philips Achieva 1.5 Tesla scanner. Consenting patients attended the RLUH department of radiology, normally as an outpatient and drank 150ml of pineapple juice 10 minutes before the scan. They were positioned on the scanning table in the supine position. Coronal and axial sequences were taken first with a balanced fast field echo, before a single shot radial coronal T2 weighted turbo spin echo sequence, with fat suppression. The repetition rate of the scan was 8000 milliseconds and the

time to echo was 800 milliseconds. All scans were performed during a resting expiratory breath hold. Images were again uploaded to the PACS system.

2.3.3.4 ERCP and Molecular Analysis of Pancreatic Juice

Technical Aspects of Performing ERCP at RLUH

Endoscopic Retrograde Cholangiopancreatography (ERCP) was conducted at RLUH in the gastroenterology suite by either a consultant radiologist or gastroenterologist. ERCP and endoluminal ultrasound (EUS) were often performed at the same clinical session. Analgesic throat spray was administered prior to intubation with supplementary intravenous benzodiazepine sedation and opiate analgesia as required. The patient was placed in the right posterior oblique position. The patient was intubated with an Olympus TJF-260V scope. A visual inspection of the upper gastrointestinal tract was undertaken as the scope was passed through the stomach into the duodenum with duodenal juice collected by aspiration and placed in a sterile universal receiver which was stored on ice at 4°C. The ampulla of Vater was then located and cannulated without administration of contrast. The cannula was aspirated with a 10ml syringe and bile and pancreatic juice aspirated. These were again placed in sterile universal receivers, labelled and stored on ice, normally by the EUROPAC fellow, before being taken to the Division of Surgery and Oncology primary lab for processing. Images of the biliary tree could be acquired digitally at the request of the gastroenterologist or radiologist during the procedure in the antero-posterior plane. This was performed using a Philips MultiDiagnost Eleva machine

which pulsed x-rays at a rate of 3 per second, although the use of contrast was minimised to reduce the chance of post-procedure pancreatitis. The consultant gastroenterologist or radiologist responsible for conducting the ERCP issued a written report using Unisoft software. The participant was then observed in the department of endoscopy recovery suite.

Technical Aspects of Molecular Analysis of Pancreatic Juice

These methods are those of the School of Cancer Studies as set out in the Yan paper[220]. In practice, the methods described were almost always carried out by Dr Yan in person, although I performed aspects of these methods, particularly *Tp53* analysis alongside Dr Yan during the first year of my research period.

Within two hours of collection at ERCP, the chilled pancreatic juice was transferred to sterile 1.5ml Eppendorf tubes and spun at room temperature in a micro centrifuge (Spectrafuge, Labnet) at 14000rpm for 5 minutes. The supernatant was aspirated and then transferred to a sterile 1.5ml Eppendorf. This was immediately stored at -80°C along with the pellets, prior to DNA extraction, with details stored on the Division of Surgery & Oncology database.

When samples were selected for testing, they were transferred into a freshly autoclaved 1500μl Eppendorf microcentrifuge tube (Eppendorf) in a laminar flow hood. Equal volumes (200μl) of sterile water and Phenol: Chloroform: Isoamyl Alcohol (25:24:1) were added to 200μl of pancreatic juice and the mixture was vortexed for 30 seconds. The aqueous and organic phases were separated by

centrifugation at 14000 rpm for 10 minutes. DNA was precipitated overnight at 4°c using 2.5 volumes of 100% ethanol and 20μl 3M sodium acetate (pH 6.8). The following day, the sample was spun at 17,000rpm (Sorval centrifuge) for 30 minutes at 16°C. The DNA was then resuspended by the addition of 200μl of molecular grade water and immediately stored at –20°C.

K-RAS Analysis

Amplification Refractory Mutation System (ARMS) Analysis for *K-RAS2* Mutations was used. This is a mutation specific PCR assay controlled with amplification of an overlapping sequence with mutation independent primers. The principle of the ARMS system involved the use of primers specific for the different *K-RAS2* mutations at codon 12 at their 3' end. The use of a mutation specific primer and a complementary primer to a downstream region of the gene resulted in amplification of a *K-RAS2* sequence.

Real-time PCR was performed using a Roche lightcycler. Initially the system used relied on air to transfer heat to samples held in glass capillaries, permitting 30-40 amplification cycles over 20-30 minutes. Subsequently the Lightcycler 480 system was used, a 96 well system with a peltier system to allow even heat exchange across the plate. In both systems the reaction mixture contained both FastStart Taq DNA polymerase and DNA double-strand specific SYBR Green I dye for detection. The FastStart Taq DNA polymerase is a modified form of thermostable recombinant

Taq DNA polymerase and was inactive at room temperature. The enzyme was 'activated' by high temperature (95°C for 10 minutes).

The function of the SYBR Green I dye was to bind to the groove of the DNA double helix in the amplified PCR products. This DNA binding enabled SYBR Green I molecules to emit light on excitation. The amplicon was detected by its fluorescence. Before amplification, the reaction mixture contained denatured DNA, the primers, and the dye. The sample for analysis being made up to 20μl using the LightCycler-DNA Amplification Kit SYBR Green I (Roche) in the following proportions: 12μl PCR grade water, 2μl Magnesium Chloride (20mM), 2μl SYBR Green I, 2μl oligonucleotide primer and 2μl of DNA (or water for negative controls). Undiluted samples were quantified by using 1μl of the DNA with 1μl of each of the control primers (concentration 20pmol/μl).

The six most common *K-RAS2* mutations result in changes from the amino acid glycine at codon 12 to another amino acid. These are named in this thesis according to the substituted amino acid, for example a change from glycine to aspartate will be termed *Aspartate*. Primers specific for the six recognised point mutations (Aspartate, Arginine, Valine, Serine, Alanine and Cysteine) were used. Real time PCR was performed after the initial activation step of 10 minutes at 95°c. This was followed by 60 cycles of: 2 seconds at 95°c, 20 seconds at 61°c, 20 seconds at 72°c and 10 seconds at 81°c. This was followed by a melting curve ramp from 72 to 95°c over 10 minutes.

Melting curve analysis was performed to verify that the PCR products were pure. This was done by increasing the temperature from 72 to 95°C at a rate of 0.04°C/sec. As the PCR products were heated and reached their respective melting temperatures, the two DNA strands dissociated. This could be detected by the rapid loss of fluorescence. The melting temperature was determined by the product length and its G/C content. As PCR product increased in length and the G/C content rose, so did the melting temperature. Analysis of melting curves revealed whether pure PCR products were present. This was indicated by a single PCR product with a single melting curve having a narrow peak. Primer dimers melted at relatively low temperatures and had broader peaks.

Quantification analysis was performed using the lightcycler software (Version 5.32) for the presence of a PCR product. The quantification value achieved using the control primers were compared graphically with the values for each of the relevant mutant-specific primers. The assay had been calibrated on both machines using varying concentrations of normal blood DNA with at least 100 pairs of PCR reactions for each mutation specific primer. This permitted the production of 98% confidence intervals on a linear or polynomial (dependent on the mutation specific primer) regression curve. Samples were analysed in triplicate for each *K-RAS2* mutation and threshold cycles plotted. If all three points were below the 98% confidence limit for the wild type only regression, the sample was considered to contain mutant sequences; otherwise it was classified as wild type.

p16 Analysis

Real time PCR measurement of *CDKN2A* Promotor Methylation in pancreatic juice was performed following modification of the DNA from pancreatic juice with sodium bisulfite[313]. Real-time PCR amplification was carried out in 20 µl volumes with 2 µl of Light Cycle DNA Mastermix, with a primer concentration of 1µM. PCR reactions were carried out on the lightcycler amplification and detection system (as above for *K-RAS2* analysis).

The mixture was pre-incubated at 95°c for 10 minutes. DNA was then amplified for 60 cycles of 20 seconds at 95°C, 10 seconds at 67°C for methylated primer or 65°C for unmethylated primer and then 10 seconds at 72°C. Data were analysed using quantification program software. The result was expressed as a methylation index (%) which was calculated using the equation below:

Methylation index = [M/(M+U)] x 100%

where 'M' was the quantity of methylated *CDKN2A* sequences measured by methylation specific PCR (MSP) following bisulfite conversion and 'U' was the quantity of unmethylated *CDKN2A* sequences measured by real-time MSP following bisulfite conversion[314].

p53 Analysis

The *Tp53* assay is a yeast functional assay system based on principles developed by Flaman *et al*[315]. The process has been modified to use genomic DNA rather than

ribonucleic acid (RNA). Exons five to eight containing the mutation hotspots[316] were selected as they coded for the DNA binding domain of the p53 protein and account for the majority of recorded *Tp53* mutations identified in cancer.

Genomic DNA was amplified by polymerase chain reaction (PCR); the conditions were 95°C for 12 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for one minute. The PCR mixture contained 0.25 units of pfu DNA polymerase, 1x pfu buffer (Promega) with 2mM Magnesium Sulphate, 20 pmol of each primer and 0.2 mm dntps. Primers were designed to allow PCR linkage of exons 5-6 and exons 7-8 and then to link 6 to 7. If possible the overlapping primers were used to amplify the exons directly from genomic DNA; otherwise an external (intronic) primer was used to give product followed by a second nested PCR.

The reporter yeast strain yig-397 was co-transformed with the PCR product of *Tp53* exons 5 to 8 and a circular expression vector carrying the full length coding sequence for p53 (pls76). The vector *Tp53* sequences were replaced by PCR amplified sequences in vivo by homologous recombination.

Wild type p53 allowed expression of ADE2 and therefore, led to production of white yeast colonies. Mutant *Tp53* sequences resulted in no expression of ADE2 and so red colonies became visible. After 48 hours incubation on selective plates (no leucine and low adenine), white and red transformants appeared. The uncut circular vector was used rather than gap repair. This had the advantage that although it decreased the ratio of red to white colonies, it reduced the number of false positives.

Previous studies showed that the p53 functional assay of tissue containing wild-type *Tp53* gave 5-10% red colonies with the gap repair vector method[315, 317]. This was due to PCR error or the self-ligation of the vector, combined with the presence of alternative splice forms of *Tp53* in the source material[315, 318]. Mutations were identified by taking plasmids from three red yeast colonies by alkaline lysis and transforming these into *E coli* DH5α and sequencing the product. If two sequences from different yeast colonies carried the same *Tp53* mutation, the sample was classified as mutant.

2.3.3.5 Technical Aspects of Processing of Pathological Specimens

Any detected abnormality was discussed at the MDT and resection would be offered if it was deemed to be in the patient's best interest. At the completion of the procedure, the fresh pancreatic tissue was collected from theatre and taken to the department of pathology where it was given to one the of the consultant pathologists with a special interest in pancreatic work. The specimen was then opened and sliced, with fresh tissue samples given to the research fellow for snap freezing in liquid nitrogen. The remainder of the specimen was then pinned to a cork board and submerged in formalin to fix for 24 hours. It was then floated off the cork board and fixed back in the formalin for a further 24 hours, before being dissected and sampled by a consultant pathologist, with sampled tissue placed into numbered cassettes. These cassettes were then returned to formalin until they went into the overnight processor, where the tissue was dehydrated through graded alcohol, put into xylene and finally wax. The next morning, the tissue was orientated into wax moulds on top

of the cassettes, hardened (by chilling the blocks on ice) and then the sections cut from these blocks. The cut sections were placed onto glass slides and put through an automated staining machine for hematoxylin and eosin staining. Alternatively, the unstained slides could go for other special stains or for immunohistochemistry.

2.3.4 Data Storage and Analysis

Results of screening investigations for participants screened at the Royal Liverpool University Hospital were obtained from hospital computer systems and from the department of gastroenterology. Where screening was performed by another centre, results were obtained by requesting hard copies from the collaborating clinician. This system was supplemented by visiting satellite sites as necessary. Data were entered and stored on the main Progeny database with the same restrictions and safeguards described in section 2.1.3.

2.3.5 Prospective Audit

The results of screening investigations were collected and presented quarterly at meetings of the EUROPAC study group. Data were presented graphically wherever possible. Continually reviewing the results led to the early identification of trends and adverse events that will be described in the results section.

2.3.6 Cost Effectiveness

Calculations regarding costs of the secondary screening study were based upon the methods used in the paper published on screening in PJS[300]. Costs will have changed slightly in the past four years but using the same values permits simple comparison with that paper. The cost of an EUS was taken as $590, the cost of an ERCP was taken as $740, with the cost of a CT scan taken as $268. MR scans are slightly more expensive than CT scans but for the intents of the cost analysis in the

results of this thesis, the cost of all cross sectional imaging investigations was taken to be $268 per investigation.

3 Results

3.1 Primary Screening

This thesis will primarily use data obtained on high risk individuals between 1997 and July 2008. I had primary responsibility for entering these data in a period between October 2006 and July 2008 with the assistance of Mr Matthew Harcus, who was appointed as the EUROPAC database manager in November 2006. Data on HP families up to January 2009 will also be included in the thesis as they formed part of a publication which I completed after the end of my official tenure as the EUROPAC research fellow.

Prior to October 2006, a total of 709 files had been opened either on the basis of enquiries or actual families recruited. Files were only opened once written consent had been obtained from the proband. During my tenure of 22 months as the EUROPAC research fellow, 114 new FPC or HP families were recruited and data held on existing families were consolidated. The HP kindreds had been recently updated by my predecessor as part of his thesis. Letters and follow up questionnaires were sent to all consenting members of the FPC kindreds during my tenure. Data obtained from primary screening will be displayed in the following sections, starting with the FPC arm of the registry and then the HP kindreds.

3.1.1 FPC Database Summary (July 2008)

By July 2008 a total of 325 families had been recruited to the FPC arm of the registry. These families contained over 8000 individuals and over 700 reported cases of pancreatic cancer. Blood DNA samples were held locally for over 500 individuals including almost 100 affected individuals. Of the 114 families recruited during my tenure, 69 were added to the FPC arm of the registry. As recruitment and the initial primary screening process was completed for each kindred, a committee which invariably included Dr William Greenhalf and myself, supplemented where possible by clinical geneticists, other clinicians and scientists (as given in the acknowledgements) classified each family. The classification system for first the FPC and then HP arms of the registry will be explained over the following pages.

3.1.1.1 FPC Families Classified by Kindred Type

Families that met the criteria for designation as 'FPC' had at least two pancreatic cancers in at least two generations. A decision was made as to whether a family was consistent with autosomal dominant inheritance. This left some families which were categorised as 'FPC query' (*FPC?*) where the precise criteria were not met, e.g. either the cancers were not in multiple generations or only one cancer case could be confirmed. A number of families had been recruited where at least two pancreatic cancers coincided with at least one gastric cancer case. These may, in time, be shown to belong to an as yet undefined syndrome, which we have given the working definition of 'With Gastric', but at present there are too few data for a publication. Prior to my participation with the EUROPAC registry, the group had discovered the

BRCA2 mutation in some families consistent with FPC. During my tenure, I was involved in organising *BRCA2* testing of further families. *BRCA2* testing was only performed if patients had given explicit consent for any genetic analysis to be reported back to them. Preliminary sequencing for purely research purposes was carried out by Drs Earl, Niemczyk, or Yan, but any reported result had to be carried out in an accredited clinical genetics laboratory. Where the *BRCA2* test showed a mutation in one of the pancreatic cancer cases and the criteria for designation as a FPC kindred were fully met, the family was given the designation '*BRCA2* FPC'. There were other families, which were not consistent with FPC, but contained single cases of pancreatic cancer with one or more cases of breast or ovarian cancer. Some of these were tested for *BRCA2* mutations either by ourselves or collaborating groups. Where *BRCA2* mutations were identified, these were classified as *BRCA2* Breast Ovarian *(BRCA2* BOv*)*. Where no mutation was identified, these families were classified simply as Breast Ovarian (BOv). The EUROPAC registry took a deliberate policy decision not to recruit families with any cancer syndrome other than FPC onto the registry. However, because the process of recruitment was progressive, some families were recruited to the study and the nature of the genetic risk within the kindred only became clear during the process of primary screening. This included a number of Breast Ovarian families that had already been recruited before my tenure. The association of pancreatic cancer with multiple cases of melanoma has been well described[319]. Initially I intended to study families that we believed carried *CDKN2A (p16)* mutations but had a phenotype consistent with FPC rather than FAMMM. However, early in my research a collaborating group (FaPaCa)

published a paper[94] indicating that *CDKN2A (p16)* mutations in pancreatic cancer families were exclusively seen in families with a predisposition to melanoma. I subsequently confirmed this observation in our families, but a number of FAMMM families are still held on the registry. The association of HNPCC and pancreatic cancer was one of the earliest defined inherited predispositions to pancreatic cancer[107]. EUROPAC has also encountered many HNPCC families during our recruitment. These include a number with proven genetic mutations (*MLH1, MSH2* or *MSH6*). We have also identified other families with suspected FPC which the classification committee decided were suspicious for HNPCC but where genetic testing was not possible. These were classified as 'HNPCC?'. As described in the introduction, neurofibromatosis (NF) is not typically associated with cancers of the exocrine pancreas. EUROPAC has identified two NF families which have confirmed cases of pancreatic cancer and this interesting finding will be further investigated by my successors. We have also included a single family with Peutz-Jeghers syndrome (PJS) on the registry. PJS is normally associated with single cases of pancreatic cancer (because of the very high risk of other forms of cancer in these families). The final group is called 'Other'. This includes the families, which for any reason, do not fulfil the criteria for classification into any of the above groups. The full breakdown of pancreatic cancer families is shown overleaf in table 3.

Table 3: Breakdown of EUROPAC FPC families by type, August 2008

Table showing the exact numbers of each type of family that had been added to the FPC arm of the EUROPAC database by July 2008. 'FPC' is the group of families that fully meet the criteria for autosomal dominance (multiple cases in multiple generations), 'FPC?' is where there are multiple cases but the specific criteria for autosomal dominance are not met. 'With Gastric' is where there at least two cases of pancreatic and at least one case of gastric cancer in a family in the absence of another causative syndrome. '*BRCA2* FPC' is FPC with a *BRCA2* mutation. '*BRCA2* BOv' is Breast Ovarian cancer syndrome with a *BRCA2* mutation and at least one case of pancreatic cancer. 'BOv' is Breast Ovarian cancer syndrome with a pancreatic cancer. Affected individuals may or may not carry *BRCA1* mutations but do not carry a *BRCA2* mutation. 'FAMMM' is at least one case of pancreatic cancer in the presence of either multiple cases of malignant melanoma or proven *CDKN2A (p16)* mutations. 'HNPCC' includes families with a phenotype consistent with the diagnosis with one of the established HNPCC genetic mutations (*MLH1, MSH2* or *MSH6*). 'HNPCC?' is families where there is a suspicion of HNPCC based on a phenotype of multiple cases of colorectal cancer, but the testable genetic mutations could either not be proven or are not present. The two 'NF' families are proven neurofibromatosis with at least two cases of pancreatic cancer and there is a single confirmed Peutz-Jeghers family. There are 45 other additional registered families. These include the full range of families that for one reason or another were recruited but were never classified as belonging to one of the other groups. Total numbers registered by July 2008 are given along with the number (in parenthesis) recruited during my period of research.

Table 3: Breakdown of EUROPAC FPC families by type, August 2008

Family diagnosis	Families	Percentage (%)
FPC	153 (35)	47 (51)
FPC?	70 (15)	21 (22)
'With Gastric'	13 (1)	4 (1)
BRCA2 FPC	5 (1)	2 (1)
BRCA2 BOv	3 (1)	1 (1)
BOv	21 (4)	6 (6)
FAMMM	3 (0)	1 (0)
HNPCC	3 (1)	1 (1)
HNPCC?	6 (3)	2 (4)
NF	2 (0)	1 (0)
Peutz-Jeghers	1 (0)	0 (0)
Other	45 (8)	14 (13)
Total	325 (69)	100 (100)

3.1.1.2 FPC Families Classified by Number of Cancer Cases

One of the most important ways of classifying FPC kindreds is by the number of cancer cases they contain. The number of cancers in EUROPAC FPC kindreds is displayed in figure 5. Clearly this is a dynamic measure. The FPC families were followed up and prospective cancers added to the database during Spring 2008, but the results will have changed again since August 2008 as further prospective cancer cases have occurred and more information on previously unidentified cancer cases within families has become available. The data shown were correct at the end of July 2008.

Figure 5: The Number of Pancreatic Cancers in EUROPAC's FPC, ?FPC, *With Gastric* & *BRCA2* FPC Families (July 2008)

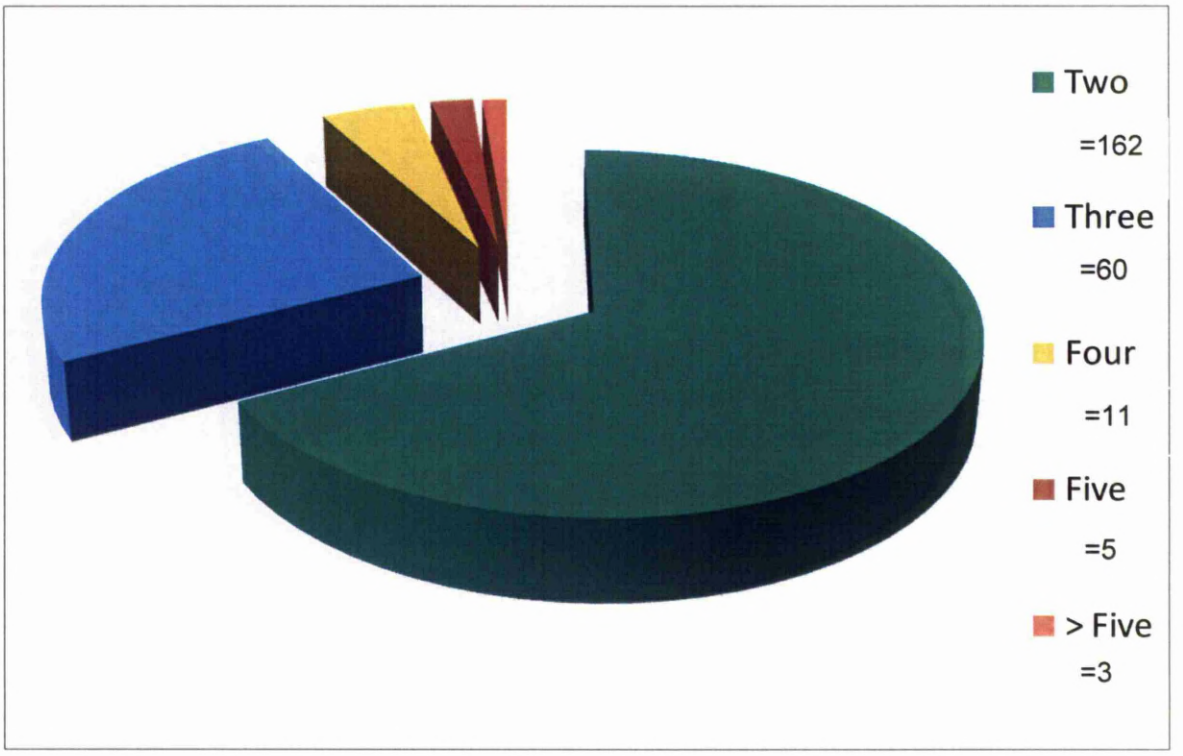


Figure showing a breakdown of the total number of FPC, ?FPC, 'With Gastric' and *BRCA2* FPC kindreds that contain the number of pancreatic cancers shown in the legend. There are 162 families with 2 cancer cases(67%), 60 (25%) with 3 cancers, 11 with 4 (5%), 5 with 5 cases (2%) and single families with 6, 8 and 10 cancer cases.

3.1.1.3 FPC Families Classified by Nationality

As EUROPAC has developed an increasingly international and collaborative approach to research, the numbers of families recruited outside the UK has increased. Whilst most of the recruited FPC families still originate from the UK, there are now significant numbers of families that have been recruited by collaborating clinicians, particularly the Clichy and FaPaCa groups in France and Germany. Others have been recruited on a piecemeal basis through individual collaborators primarily within Europe, but also further afield. As previously stated US families are redirected to the US registries. A breakdown of the FPC database by nation of origin is shown below. Families recruited from overseas tend to contain higher numbers of cancers. This is probably due to recruitment bias, with only the most severely affected families being referred and consenting to join an overseas registry. All the families with more than five pancreatic cancers were recruited by non-UK collaborators.

Figure 6: A Breakdown of EUROPAC FPC, ?FPC, 'With Gastric' & *BRCA2* FPC Families by Nationality, July 2008

Figure showing the country of origin of families registered with EUROPAC and meeting the criteria for designation as a FPC, ?FPC, With Gastric or *BRCA2* FPC kindred in July 2008. The vast majority obviously come from the UK. The French and German families were contributed by the Clichy and FaPaCa groups respectively, with the remainder coming in small numbers from other international, predominantly European, collaborators.

3.1.2 HP Database Summary (July 2008)

By July 2008 over 450 families had been recruited to the pancreatitis arm of the EUROPAC database. These families included almost 7000 individuals of which over 1100 either reported, or were reported to have had symptoms of pancreatitis; there were 76 confirmed cases of pancreatic cancer. Participants were consented for genetic testing and DNA was sent to the Mersey Regional Genetics Service at the Liverpool Women's Hospital. Testing was specifically aimed at identification of the p.R122H, p.N29I and p.A16V mutations of the *PRSS1* gene and so sequencing was initially limited to exons one, two and three of that gene. If none of these mutations were identified, the remainder of the gene was then sequenced.

3.1.2.1 HP Families Classified by Mutation and Kindred Type

Table four (see below) summarises the families registered in July 2008 that were consistent with an autosomal dominant inheritance of a predisposition to pancreatitis (at least two cases in at least two generations) as well as families with a defined mutation even where autosomal dominance had yet to be proven. Classification was normally by mutation. Where no *PRSS1* mutation was identified, but families had a phenotype consistent with HP, they were classified as 'Neg All HP'. There are some families with proven *PRSS1* mutations (or polymorphisms) other than p.R122H, p.N29I or p.A16V, which are too rare to have been clinically characterised; these fall within the group 'Other Mutations'. Finally there are some families included in this table, where genetic testing was not performed during my period of research. Seven

of these were families in whom testing should be possible in future (HP families) and two were families where the proband has refused genetic testing but was willing for the family to remain on the registry (HP problem).

Table 4: A Summary of Primary Screening Data for HP families (July 2008)

Table showing types of true HP families broken down by number of total individuals (1st, 2nd or 3rd degree relatives of the index case); individuals affected by pancreatitis; and individuals affected by pancreatic cancer. Groups are determined by *PRSS1* mutation, with p.A16V kindreds listed in this table irrespective of phenotype. The 'Neg All HP' group includes families where the phenotype is consistent with HP but a mutation has not been identified despite sequencing of *PRSS1*. The group 'Other mutations' includes very rare *PRSS1* mutations or polymorphisms, which are too rare to have been clinically characterised. 'HP families' are kindreds where the phenotype is consistent with HP and genetic testing should be possible but has yet to be initiated or is in process. 'HP problem' is where the proband wishes to be on the registry, but has declined genetic testing. Numbers in black are the totals in July 2008, numbers in parentheses are those recruited during my time as the EUROPAC research fellow.

Group	Families	Total individuals	Affected individuals	Cancer Cases
p.R122H	98 (4)	2447 (125)	449 (10)	25 (0)
p.N29I	41 (1)	1159 (16)	177 (1)	11 (1)
p.A16V	10 (5)	272 (76)	24 (10)	3 (2)
Neg All HP	41 (5)	787 (94)	145 (23)	14 (2)
Other mutations	13 (0)	94 (0)	22 (0)	1 (0)
HP families	7 (2)	169 (49)	18 (6)	0 (0)
HP problem	2 (0)	14 (0)	12 (0)	1 (0)
HP TOTAL	**212 (17)**	**4942 (360)**	**847 (50)**	**55 (5)**

3.1.2.2 HP Families Classified by Nationality

EUROPAC was intended to be a collaborative venture from the start, but in practice was initially dominated by UK families recruited by UK collaborators. This situation changed during my tenure, initially as the result of the collaboration with the group from Nijmegen, Holland and later, the decision to collaborate with the registry at Clichy, France. The collaboration with the French also led to a further family being recruited to the study to improve characterisation of the p.A16V mutation.

The HP side of the registry remains UK dominated but figure 7 shows a much more equal balance of countries of origin of 'true HP' families than is the case in the 'true FPC' kindreds. The main area of recruitment from outside the UK is France, then Germany, with a total of 55 kindreds recruited by other collaborators from other countries. There are two families from outside Europe. One was recruited by a collaborator in New Zealand, where there is no national registry, with data for the second family having been provided by Professor David Whitcomb of Pittsburgh, USA as part of the p.A16V study. As with FPC, US families are routinely redirected to the US registries.

Figure 7: A Breakdown of EUROPAC HP families by Nationality, July 2008

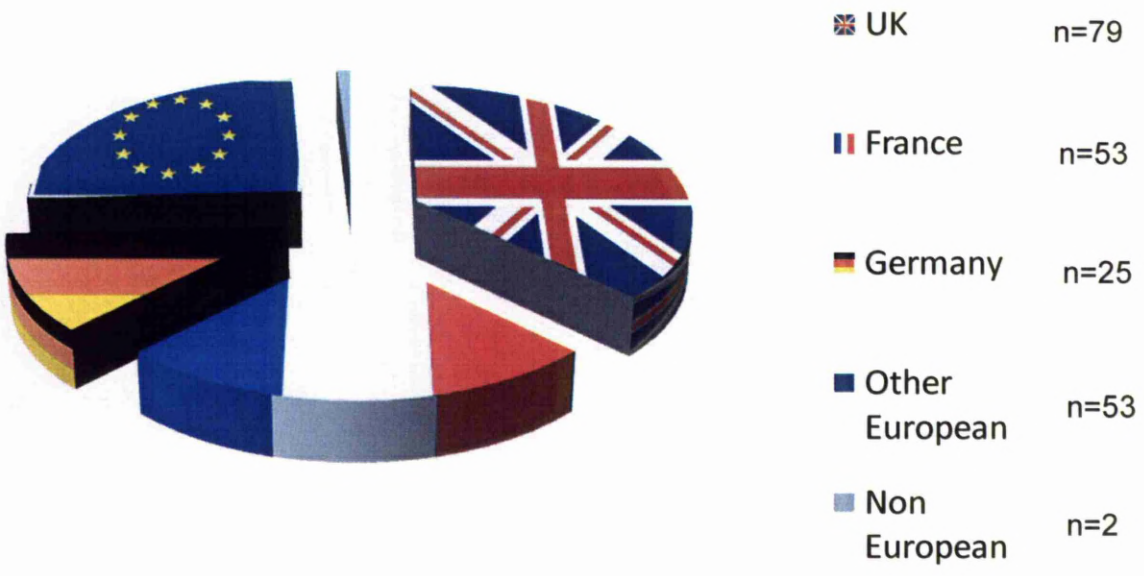


Figure showing the country of origin of families registered with EUROPAC and meeting the criteria for designation as a HP kindred by the end of July 2008. All p.A16V kindreds have been designated as HP kindreds irrespective of phenotype. The greatest number of families obviously comes from the UK, with significant contributions from both the French and German groups based at Clichy and Greifswald respectively. The remainder have been recruited via other international, predominantly European collaborators, with one family from New Zealand and a p.A16V family from the USA, recruited specifically for the p.A16V study.

3.1.2.3 Clinical Characteristics of HP

The most obvious parameters to include in quantifying risk of cancer in HP are the clinical features of the pancreatitis and the genotype. These obviously may overlap as the mutation could impact directly on the severity of the pancreatitis and thus (presumably) on cancer risk. Analysis of the clinical phenotype produced by the different *PRSS1* mutations is therefore fundamental to this thesis. Potential areas of study include the age of onset of pancreatitis and the development of endocrine and pancreatic failure. Figures for these are shown over the following pages. The existence of pancreatic failure is a factor that must be taken into account when deciding to screen an individual and will influence the balance of risk and benefit to the patient when performing a resection for suspected malignancy. Penetrance and cancer risk are also obviously relevant. It is an attractive theory that the risk of pancreatic cancer in HP is directly related to the age of onset of pancreatitis. Prior to this thesis, this had never been properly examined and the results are shown at the end of this section as figure 13. To avoid repetition a survival curve comparing risk in HP kindreds to the general population has not been included in this section, but is included below at the start of the section on risk stratification in HP as figure 22.

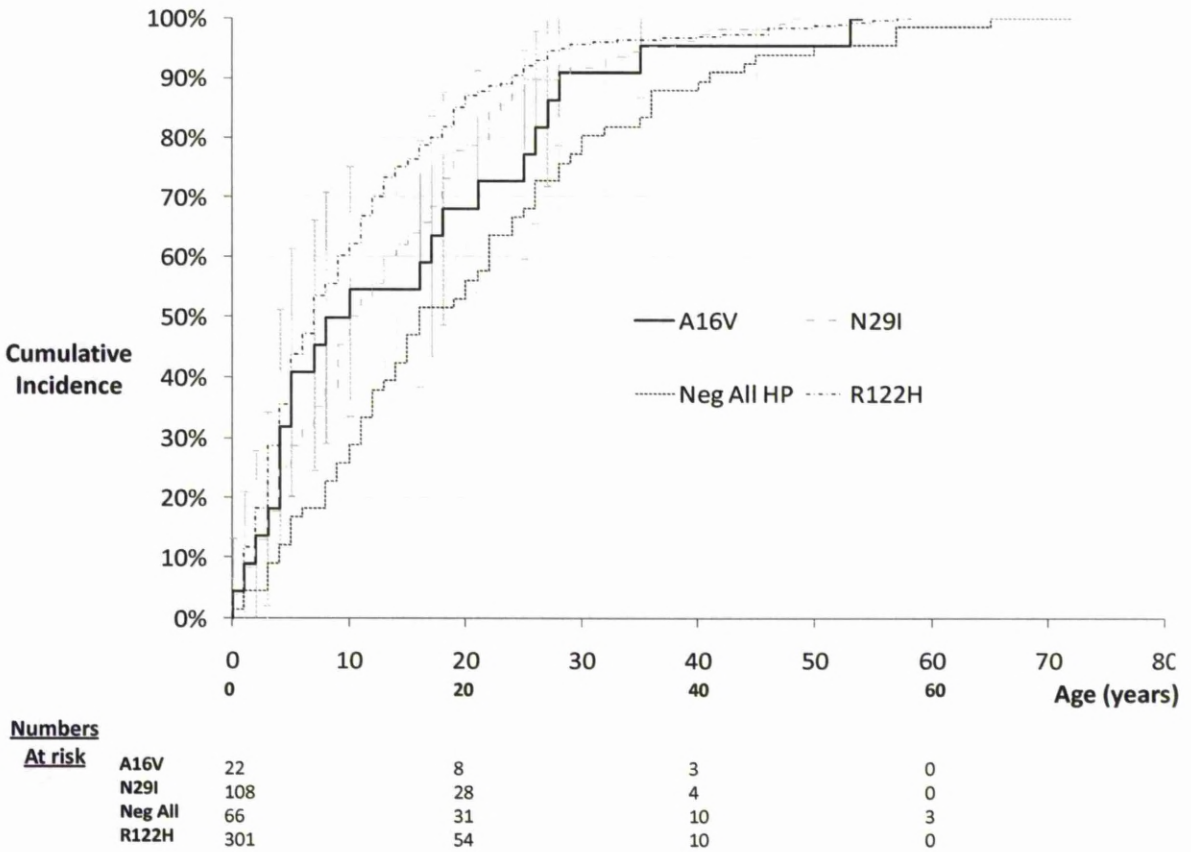
It should be noted that the data used in the rest of this section were prepared using the data guillotine used for the p.A16V study (January 2009) so these differ slightly from the July 2008 figures used in table 4.

Age of Onset of Pancreatitis

Much of the data in this section relate to the analysis performed investigating possible differences between the phenotype produced by the p.A16V mutation of *PRSS1* as compared to the more common mutations. A visual comparison of pedigrees indicates that there are differences between the phenotype of p.A16V and the phenotype associated with other *PRSS1* mutations, but the low prevalence of the p.A16V mutation and the even smaller numbers of affected individuals with pancreatitis, diabetes mellitus, malabsorption or pancreatic cancer mean that there is insufficient power for a definitive statistical analysis.

The data for onset of pancreatitis, diabetes and malabsorption for all mutation groups are shown in three separate figures over the next three pages. These data were all correct as of January 2009, when the final analysis for the p.A16V study was performed.

Figure 8: Age of Onset of Pancreatitis in EUROPAC HP Kindreds (Jan 2009)

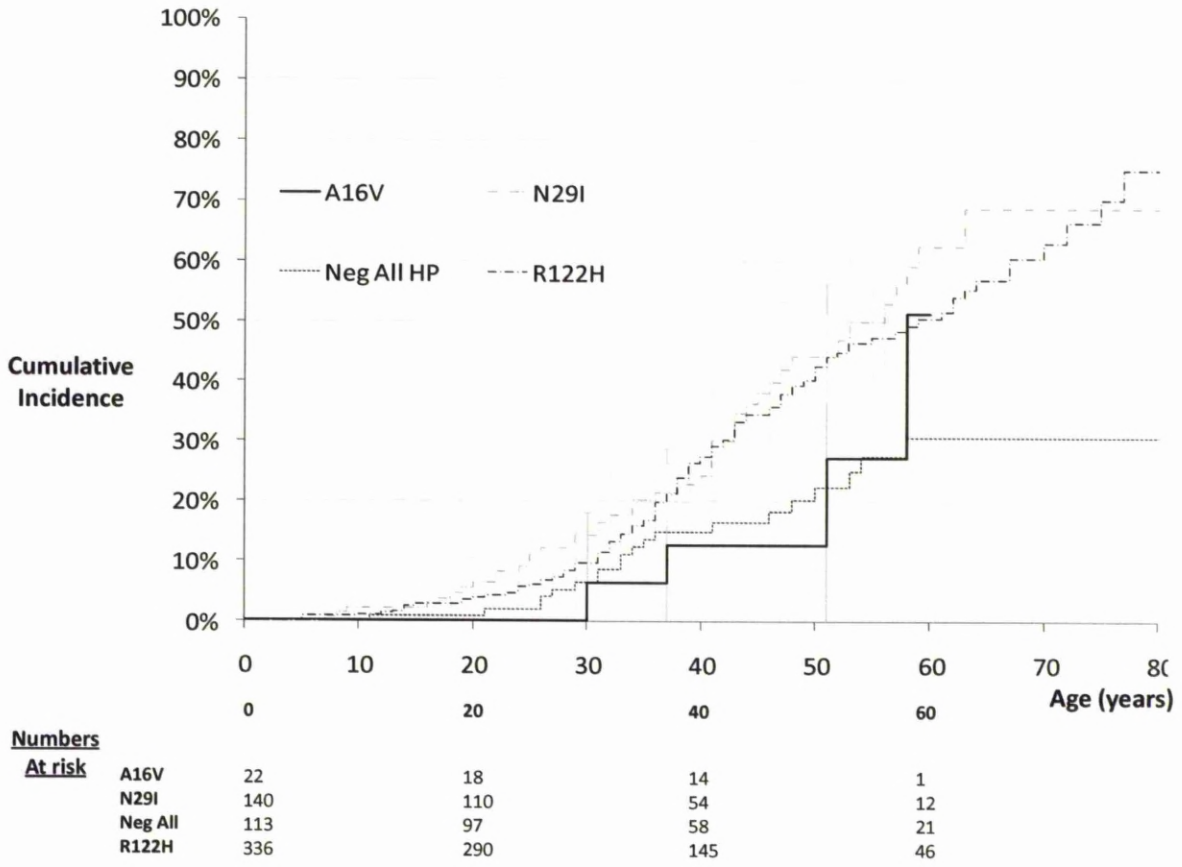


Numbers At risk					
	A16V	22	8	3	0
	N29I	108	28	4	0
	Neg All	66	31	10	3
	R122H	301	54	10	0

Figure showing the cumulative incidence of pancreatitis in the different mutation groups p.R122H, p.N29I, p.A16V, and Neg All HP, where the phenotype is consistent with HP but no mutation has been identified after *PRSS1* gene sequencing. This figure was prepared to see if there was any statistical difference between onset of pancreatitis in p.A16V kindreds compared to the other mutation groups, but the low numbers of affected individuals in the p.A16V group mean that there is insufficient power for a definitive statistical analysis.

Age of Onset of Diabetes

Figure 9: Age of Onset of Diabetes in EUROPAC HP Kindreds (Jan 2009)

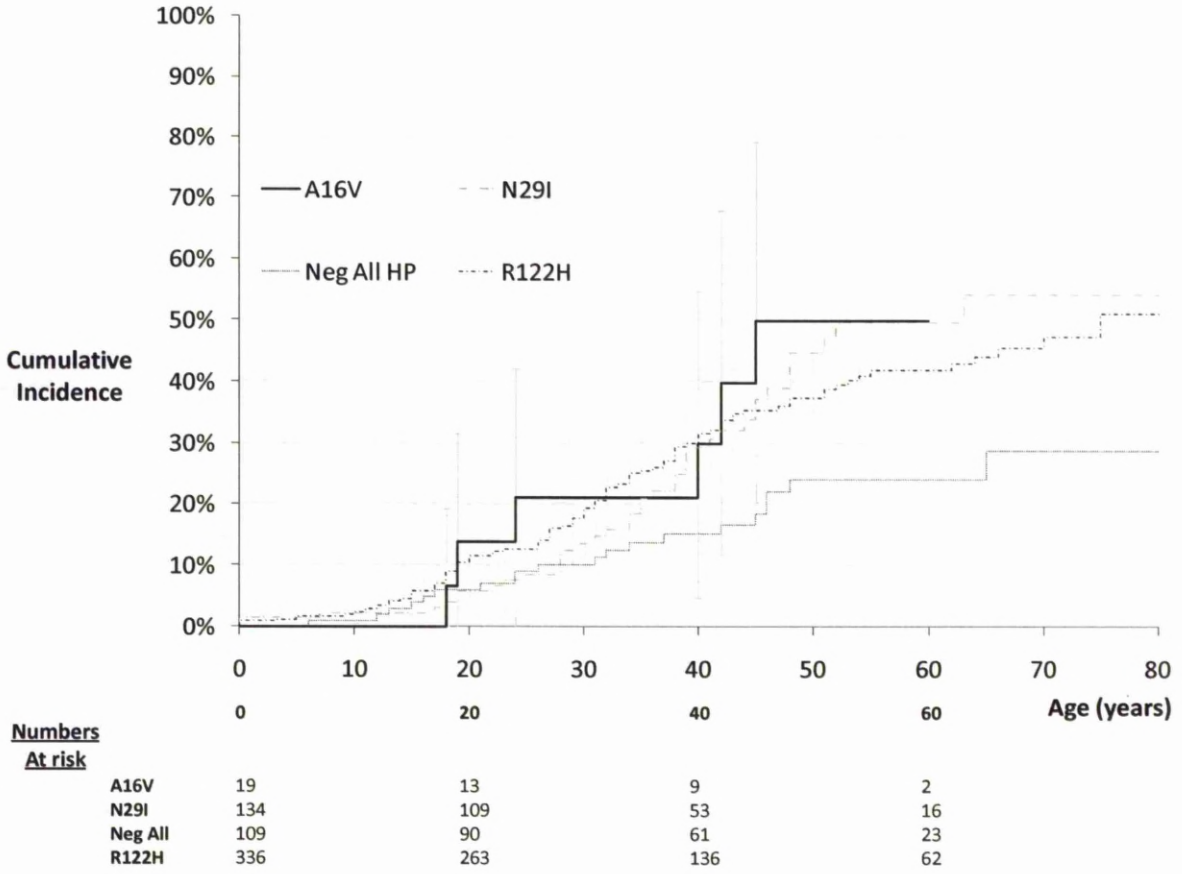


Numbers At risk	0	20	40	60
A16V	22	18	14	1
N29I	140	110	54	12
Neg All	113	97	58	21
R122H	336	290	145	46

Figure showing the number of affected individuals from HP kindreds that have endocrine pancreatic failure. This is diagnosed by raised fasting glucose or the requirement for regular prescription of either oral anti-hyperglycaemics or injected porcine insulin. Of the numbers shown, 120 affected individuals have both endocrine and exocrine pancreatic failure. Diagnosis of exocrine failure is on the basis of either a faecal elastase result below the normal range or the requirement for regular prescription of supplementary pancreatic enzymes.

Age of Onset of Malabsorption

Figure 10: Age of Onset of Malabsorption in EUROPAC HP Kindreds (Jan 2009)



Numbers At risk	0	20	40	60
A16V	19	13	9	2
N29I	134	109	53	16
Neg All	109	90	61	23
R122H	336	263	136	62

Figure showing the number of affected individuals from HP kindreds that have exocrine pancreatic failure. A diagnosis of exocrine failure is accepted on the basis of either a faecal elastase result below the normal range or the requirement for regular prescription of supplementary pancreatic enzymes. Of the numbers shown, 120 affected individuals have both endocrine and exocrine pancreatic failure. Diagnosis of endocrine failure is by raised fasting serum glucose or the requirement for regular prescription of either oral anti-hyperglycaemics or injected porcine insulin.

For all end points 'Neg All HP' is significantly different from the two groups with known HP mutations (p.R122H and p.N29I). These differences have already been described and discussed elsewhere in previous publications[92, 93]. There are too few data to prove differences between p.A16V and the other groups, but the survival curves illustrate possible trends. To simplify analysis and increase power, the two mutation groups (p.R122H and p.N29I) were combined and will be described as the 'mutation group' in contrast to the 'Neg All HP' group.

The age of diagnosis of diabetes appears similar in p.A16V and the 'Neg All HP' group (Logrank *P* value for p.A16V compared to the combined mutation group was 0.076, with a *P* value for p.A16V compared to the 'Neg All HP' group of 0.83). In contrast, when looking at age of diagnosis of exocrine pancreatic failure, the p.A16V group is more like the mutation group than the 'Neg All HP group' (Logrank *P* value for p.A16V compared to the 'Neg All HP' group is 0.087 with a *P* value for p.A16V compared to the combined mutation group of 0.88). There is the suggestion that the age of onset of pancreatitis in p.A16V appears later than with the mutation group and is more like the 'Neg All HP' group than either p.R122H or p.N29I (Logrank *P* value for p.A16V compared to the combined mutation group is 0.080, $\chi^2_1 = 3.06$, contrasting with a *P* value for p.A16V compared to the 'Neg All HP' group of 0.18).

Penetrance of the *PRSS1* Mutations

Estimates of penetrance so far have focussed on kindreds where the responsible *PRSS1* mutation is generally one of high penetrance. Families have samples taken from as many individuals as possible within the kindreds, with the total of affected individuals expressed as a percentage of the total number of mutation carriers identified. As the penetrance of a mutation reduces, an exact calculation of penetrance becomes more difficult as individuals that are not affected are often reluctant to agree to genetic testing. As part of the p.A16V study and this thesis an alternative mathematical approach was developed. The index case in each family was identified. The proportion of this index case's first degree relatives affected by pancreatitis was calculated by dividing the number of first degree relatives affected by pancreatitis by the total number of first degree relatives. Assuming an autosomal dominant pattern of inheritance, each first degree relative of an affected individual has a 50% chance of being a mutation carrier, so the derived figure was doubled to give an estimate of penetrance. This was performed blind to any mutation analysis using all families on the EUROPAC registry with the results shown in Table 5. Not all the families used in this analysis had a phenotype consistent with autosomal dominant inheritance. Where all the pancreatitis cases in the family were within a single generation (suggesting a multigene or environmental cause), these families were termed Single Generation (FIP). All p.A16V families were grouped together irrespective of phenotype.

Table 5: Estimate of Penetrance in Pancreatitis Families

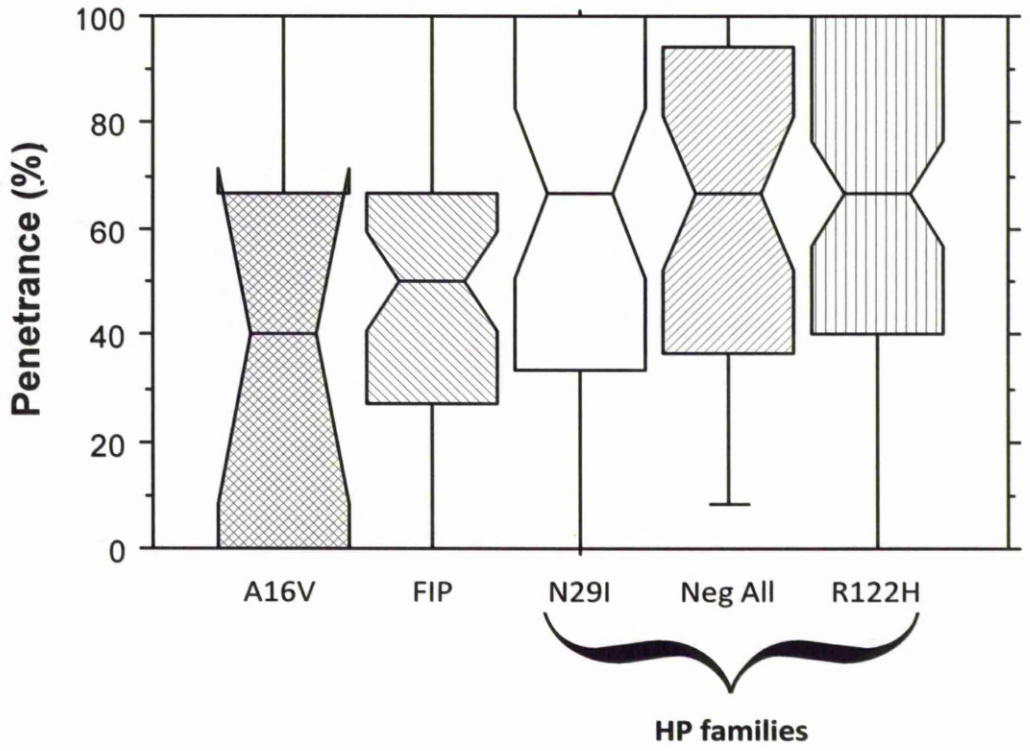
A summary of the total number of first degree relatives of the index case in each mutation or phenotype group and the number that are affected by pancreatitis. Assuming that 50% of all first degree relatives are mutation carriers, the result can be doubled to give an estimate of penetrance in each group. Families are grouped by *PRSS1* mutation. There are some families (Neg All HP) with a phenotype consistent with HP but no identifiable *PRSS1* mutation despite sequencing of the gene. The group 'Single Generation (FIP)' contains families where all the pancreatitis cases occur within a single generation. All p.A16V families were grouped together irrespective of phenotype, the breakdown of this group being shown in table 11 of this thesis.

Mutation	First degree relatives of the index case	First degree relatives affected by pancreatitis	Estimated Penetrance (if 50% of first degree relatives are carriers)
p.R122H	448	157 (35.0%)	70%
p.N29I	189	57 (30.2%)	60.4%
Neg All HP	210	66 (31.4%)	62.8%
Single Generation (FIP), excluding p.A16V	218	43 (19.7%)	39.4%
p.A16V (All groups)	62	14 (21.9%)	43.8%

This analysis is subject to ascertainment bias. Families with greater numbers of cases are more likely to be recruited. Furthermore, in families with variable penetrance, an index case is more likely to be in a section of the family with the greatest number of affected individuals. On the other hand, this approach also means that one affected carrier is always omitted from the calculation (the index case). In Figure 11 (see below), the penetrance for individual families is represented in a box plot. Kruskal-Wallis testing of the medians showed a significant difference across the five groups with a *P* value of 0.02, although p.A16V was not significantly

different from any of the other 4 groups if tested with the Mann Whitney U-Test (*P=0.06 to 0.8,* going from right to left in Figure 11).

Figure 11: An Estimate of Penetrance in Different Mutation Groups



Penetrance for individual families is estimated by doubling the proportion of affected first degree relatives of index cases. This assumes that 50% of first degree relatives of the index case are mutation carriers. Results of actual mutation analysis and knowledge of off kindred relationships were ignored in this calculation. The box plot shows the inter-quartile range (box) and the range (whiskers). The indented region indicates the 95% confidence interval for the median (marked with a horizontal line). Kruskal-Wallis testing of the medians showed a significant difference across the five groups with a *P* value of 0.02. The medians in this figure differ from the mean (across all individuals for each group) displayed in table 5 because the data are skewed. The FIP group has some families that will have no first degree relatives with pancreatitis, once the proband has been discounted (i.e. the penetrance will be zero).

3.1.2.4 Non-Hereditary Pancreatitis Families

In 241 families shown in table 6, the EUROPAC classification committee decided that there was no convincing evidence of autosomal dominant inheritance of a predisposition to pancreatitis. The majority were simple sporadic cases of idiopathic disease but in other families, although there were multiple cases of pancreatitis, these were restricted to a single generation. EUROPAC has termed such families 'Familial Idiopathic Pancreatitis' (FIP). In nine kindreds, cases of apparent sporadic pancreatitis or FIP were associated with at least one case of pancreatic cancer. A separate classification group was established during my tenure for this, termed 'Idiopathic Pancreatitis with Cancer' (IPCA).

Prior to my time in post, EUROPAC had specifically aimed to only recruit HP kindreds, whether this was based on genotype or phenotype analysis. Any non-HP kindreds recruited prior to my tenure had been recruited unintentionally, as recruitment took place prior to the primary screening process or genetic testing. On classification, there was insufficient evidence to classify the families as true HP kindreds, but the data were retained. During my tenure, there was a deliberate change in recruitment strategy, where single cases of pancreatitis were recruited if there was evidence of a pancreatitis associated genetic mutation. These mutations might be in the *CFTR* gene, (these families were classified as CFPANC) or towards the end of my time in post, affected individuals with mutations of the *SPINK1* gene were also recruited. I also registered individuals affected by pancreatitis, where there was a related case of pancreatic cancer as described above. Changing EUROPAC's

recruitment policy has obvious implications for introducing or at least exacerbating ascertainment bias in the recruitment process, but should, in time, permit research in kindreds with *CFTR* and *SPINK1* gene mutations and permit further characterisation of their clinical phenotype, using the same methods used in the figures shown in the previous section.

Table 6: A Summary of Primary Screening Data for Families or Individuals with Idiopathic or Sporadic Pancreatitis (July 2008)

Table showing a breakdown of the number of each type of family with pancreatitis not consistent with HP. Data have been broken down by number of total individuals within the kindreds, individuals affected by pancreatitis and individuals affected by pancreatic cancer. Numbers in black are the totals in July 2008, numbers in red (in parentheses) are those recruited during my period as the EUROPAC research fellow. The kindred types include: Familial Idiopathic Pancreatitis (FIP), where there are multiple cases of pancreatitis within a single generation; Sporadic, which contains single affected individuals, including the small number of affected individuals with a proven *SPINK1* mutation recruited in the last months of my research period; IPCA, where there was one case of pancreatitis and one of pancreatic cancer; and CFPANC where there was a case of pancreatitis with a proven *CFTR* mutation.

Group	Families	Total individuals	Affected individuals	Cancer Cases
FIP	44 (11)	941 (100)	95 (21)	6 (3)
Sporadic	172 (11)	743 (125)	172 (11)	0 (0)
IPCA	9 (3)	155 (50)	9 (3)	15 (6)
CFPANC	16 (3)	172 (42)	18 (3)	0 (0)
Idiopathic Totals	**241** (28)	**2011** (317)	**294** (38)	**21** (9)

Onset of Pancreatitis and Cancer Risk

As stated above, age of onset of pancreatitis in affected individuals is interesting in its own right and it is logical to theorise that the number of years an individual has been exposed to chronic pancreatic inflammation could be related to their cancer risk. Figure 12 shows that there is a poor correlation between age of onset of pancreatitis and cancer in EUROPAC HP kindreds.

Figure 12: Onset of pancreatitis and cancer in EUROPAC HP kindreds

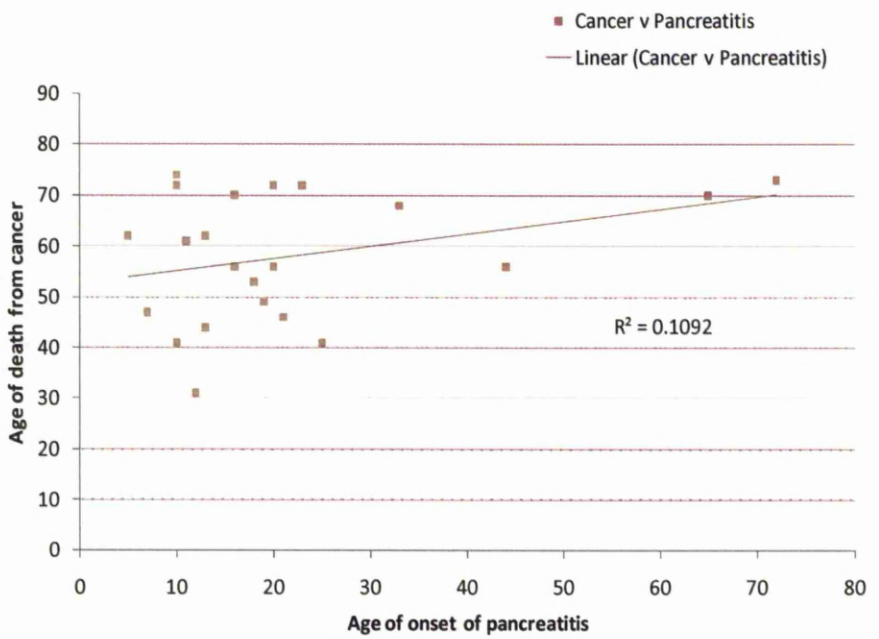


Figure illustrates the minimal relationship between age of onset of pancreatitis and age of onset of pancreatic cancer. The relationship was assessed using R^2 testing, which showed the proportion of the variability in this dataset that is accounted for by linear regression to be less than 11%.

3.2 Risk Stratification

As stated in section 1.8, improving risk stratification was the first aim of this thesis. Figure 12 shows that contrary to expectation, age of onset of pancreatitis correlates poorly with cancer risk. In contrast, other factors were identified that did impact on cancer risk and these factors have been used to develop a preliminary system of risk stratification, for both FPC and HP kindreds. These will be introduced in this section along with a description of a novel mathematical model to stratify risk in FPC kindreds, which permitted this to be performed on an individual, rather than familial basis, for the first time.

3.2.1 Risk Stratification in FPC Kindreds

Before the progress made in risk stratification in FPC kindreds is shown, it is important to see what the risk of pancreatic cancer is in these kindreds.

3.2.1.1 What is the Pancreatic Cancer Risk in FPC?

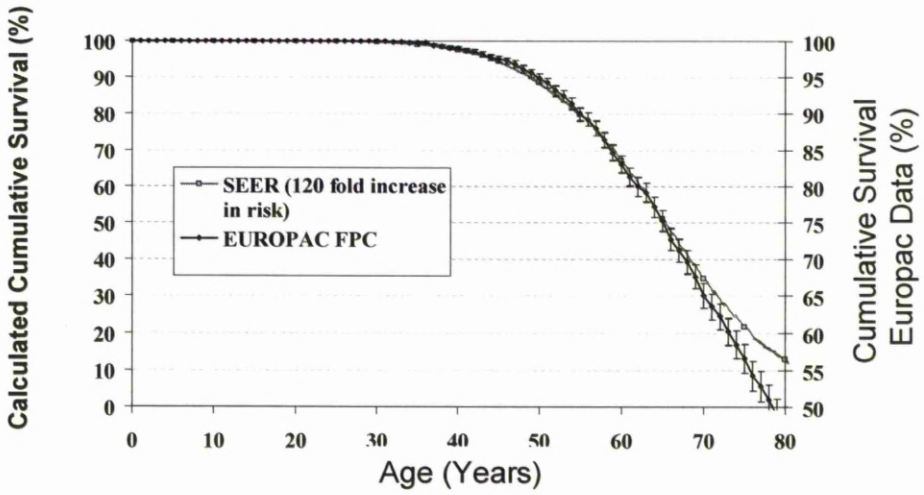
The risk from pancreatic cancer in FPC kindreds has been incompletely characterised, prompting the work set out in the remainder of section 3.2.1. As stated previously, the best available published work at the start of this thesis was that of Klein *et al*[185], where the relative risk of cancer within kindreds was expressed depending on the number of cancer cases they contained. The risk in EUROPAC's FPC kindreds has only recently been published[96], with their pancreatic cancer risk

compared against that of the general population of the United States using the SEER data.

The SEER data are cross-sectional, while familial data, such as that held by familial pancreatic cancer registries are, by definition, longitudinal. To compare the two sets of data it is either necessary to model longitudinal data using the SEER figures or to take a date for a cross-sectional study of the registry data.

This work suggested a constant increased risk for all age groups, which approximates to a 120 fold increase in risk. Even with this 120-fold increase, risk below the age of 40 is negligible, which supports that being the age of entry to the secondary screening study.

Figure 13: Age Specific Risk of Pancreatic Cancer in Families with FPC



At risk (Europac)	**2011**	**1772**	**1311**	**689**	**133**
Cumulative events (Europac)	**0**	**0**	**14**	**158**	**359**

Figure 13a: The Surveillance Epidemiology and End Results (SEER) survey in the USA has provided cross sectional data on cancer risk in five year ranges. This can be used to model longitudinal cumulative survival, allowing comparison with a Kaplan-Meier survival curve. Approximately 50% of the FPC family members are predicted to be mutation carriers, meaning baseline survival from pancreatic cancer of all potential carriers should be approximately 50%.

Figure 13b: Probability of Developing Common Cancers in the US General Population Compared to the Pancreatic Cancer Risk in EUROPAC FPC Kindreds

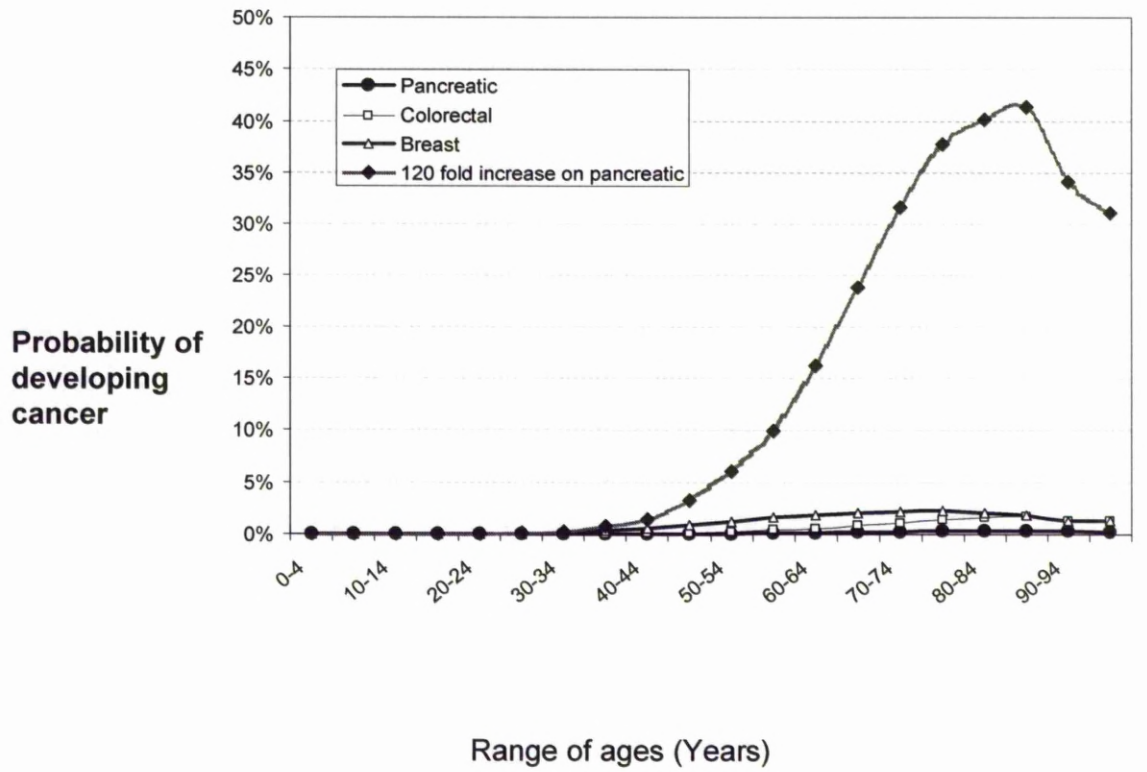


Figure 13b shows the approximate pancreatic cancer risk in EUROPAC FPC kindreds (derived by increasing the SEER pancreatic cancer risk 120-fold) compared with the risk of pancreatic and other common cancers in the US general population, which were all derived from the SEER data.

3.2.2 Methods of Risk Stratification in FPC

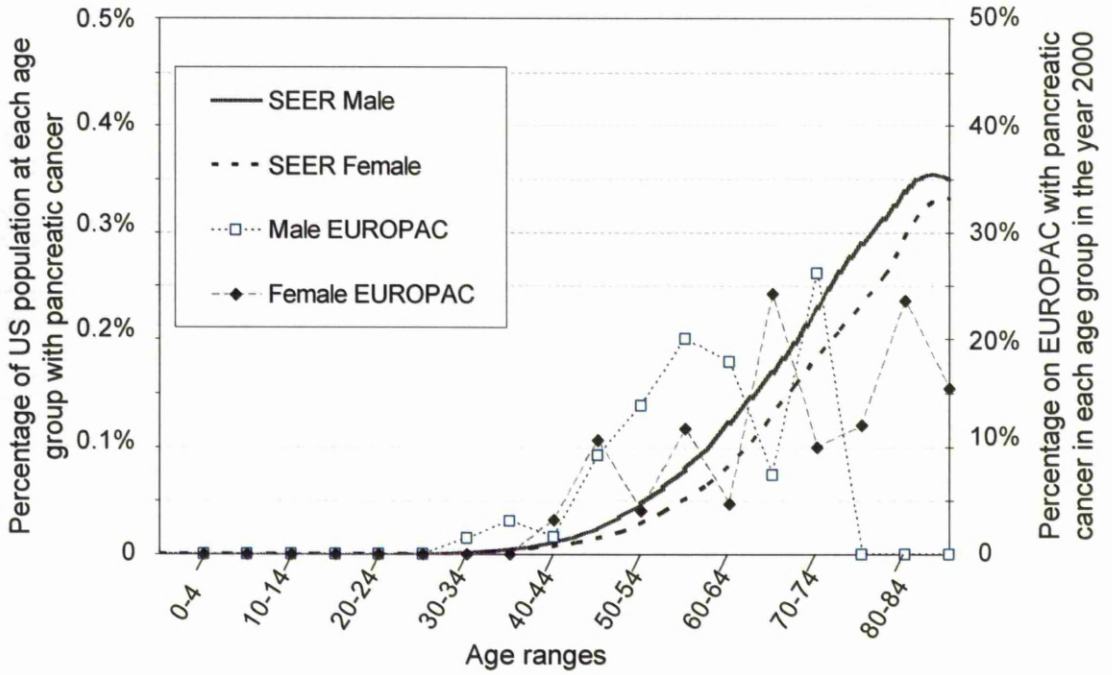
As described previously in this thesis, there are traditional methods of risk stratification in FPC, which analyse that data gleaned from traditional primary screening. There are also novel methodologies. The following section will show what can be gleaned from analysis of primary screening data before the novel methods are examined.

3.2.2.1 Analysis of Primary Screening Data

Demographics

Further analysis of the Surveillance Epidemiology and End Results (SEER) data[120] gives an insight into how risk compares between the genders showing a slightly greater incidence of pancreatic cancer in men than women. A comparison has been carried out in Figure 14a with data from the EUROPAC registry, taking individuals alive in the year 2000 and using a five year window for occurrence of pancreatic cancer. The SEER data clearly show a higher incidence of pancreatic cancer in men in all age groups. The EUROPAC data are far less clear cut, although this could be due to the small numbers of at risk individuals in each age group. Overall, in the EUROPAC families, death from pancreatic cancer does occur slightly earlier in males, (see Figure 14b) but the final lifetime risk for men and women is roughly equivalent in this population (approximately 50%), which is, again, consistent with autosomal dominant inheritance of a predisposition to pancreatic cancer and is in stark contrast to the situation in sporadic disease.

Figure 14: Gender and Risk of Pancreatic Cancer in FPC Families and Sporadic Disease



Number of at risk individuals in each age range (m=million)

Female E	36	54	60	72	63	73	42	36	17
Male E	30	53	69	68	61	58	28	23	1
Female SEER	1.4m	1.5m	1.4m	1.5m	1.7m	1.4m	0.8m	0.7m	0.5m
Male SEER	1.5m	1.6m	1.5m	1.6m	1.7m	1.3m	0.8m	0.6m	0.3m

In Figure 14a the incidence of pancreatic cancer for each gender is plotted for 5 year age groups using the SEER data. More men develop pancreatic cancer than women in each age group. This is compared to data from the EUROPAC database using a separate scale, taking the age of individuals alive in 2000 and following for pancreatic cancer until 2005. The number of individuals taken for the analysis are given below the graph (E=EUROPAC). There is a trend for a higher percentage of men to develop pancreatic cancer in the earlier age groups, but the small number in each group makes comparisons difficult.

Figure 14b: Survival of Males and Females in EUROPAC FPC Kindreds

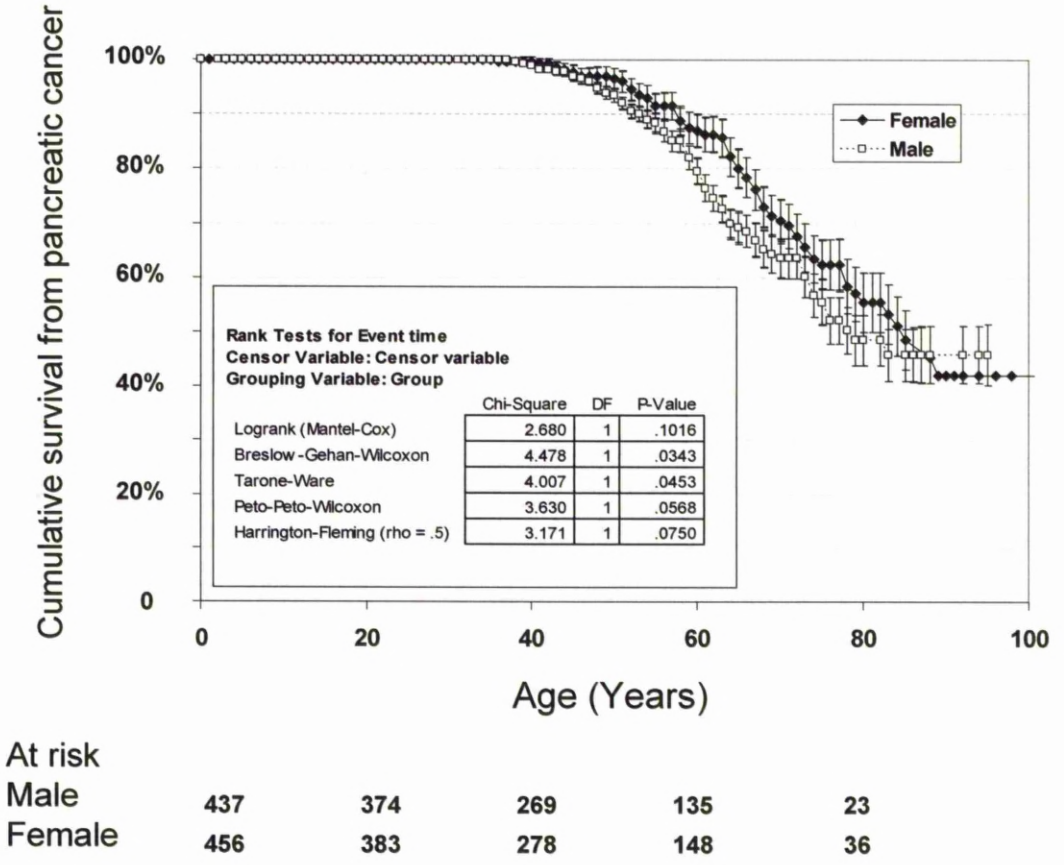
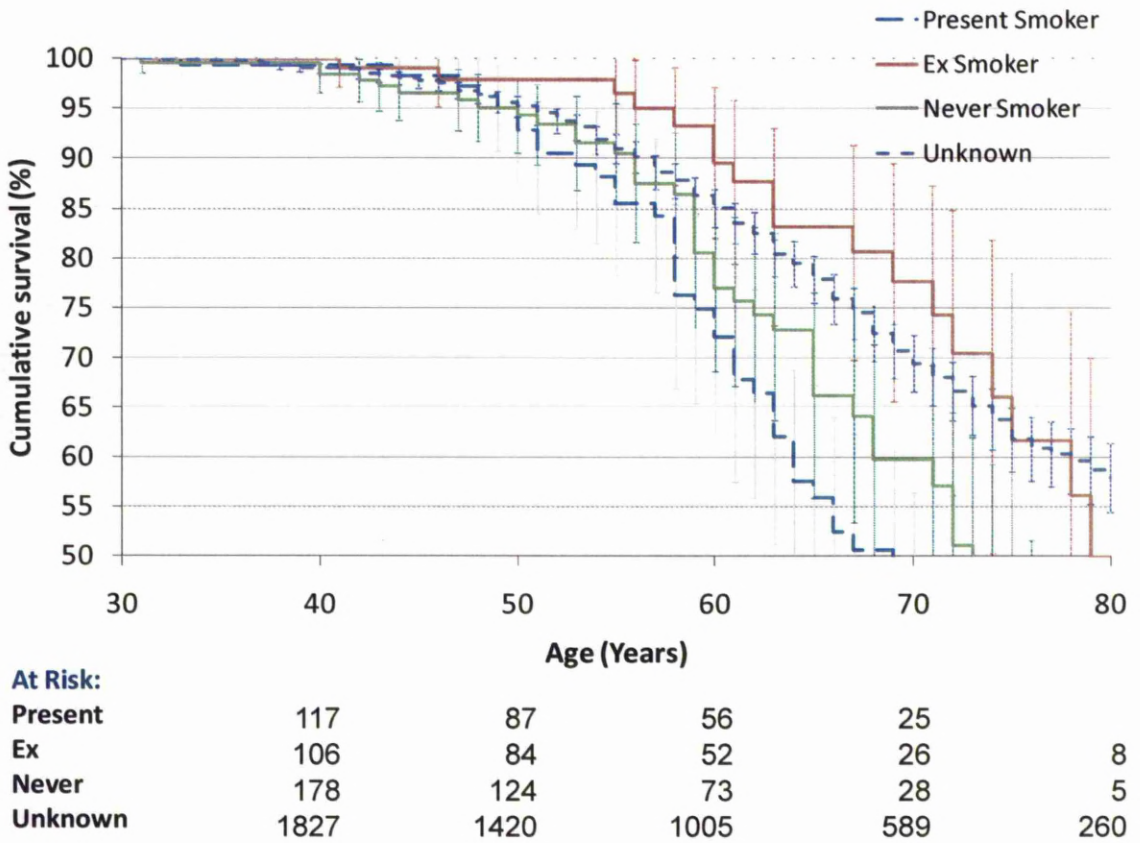


Figure 14b shows a survival curve for both males and females from EUROPAC FPC kindreds. Women develop pancreatic cancer later than men, but overall lifetime risk is equivalent.

Lifestyle Risk Factors: Smoking

The risk factor where there is the clearest link to sporadic pancreatic cancer is tobacco smoking[125]. Figures vary between the studies but the risk of pancreatic cancer has been calculated to be two-fold higher in smokers[126], with some evidence of a dose-response relationship[127]. This raises the question as to the significance of the relationship between smoking and the cancer deaths in FPC kindreds. Taking the end of July 2008 as a guillotine date (the data displayed in table 3), EUROPAC held smoking data on several hundred individuals. A Kaplan-Meier analysis was performed, with the numbers 'at risk' displayed beneath, which is shown on the following page. The results show that present smokers had the worst survival, followed by 'never smokers', with 'ex-smokers' having the best survival. The reason for this is likely to be reporting bias. Many of the data collected by EUROPAC are collected retrospectively. Family members will remember if a relative was a smoker or non-smoker at the time of their diagnosis or death, but relatives are unlikely to be able to give accurate data on whether an individual smoked at some point in the past, so an unknown proportion of the deceased reported 'never smokers' will have been 'ex-smokers'. Although it is difficult to quantify the degree of bias involved, clearly it will be in the direction of fewer 'ex-smokers' being reported within those affected by pancreatic cancer (because these individuals will almost invariably be dead).

Figure 15: Pancreatic Cancer and Smoking in EUROPAC FPC Kindreds



At Risk:					
Present	117	87	56	25	
Ex	106	84	52	26	8
Never	178	124	73	28	5
Unknown	1827	1420	1005	589	260

Figure to show the relationship between smoking and death from pancreatic cancer in EUROPAC FPC kindreds. The data show that 'never-smokers' have poorer survival than 'ex-smokers', which is likely to be the result of data bias.

3.2.2.2 Mathematical Modelling

Despite the information that can be gleaned from the statistical analysis of the primary screening data shown in the preceding section, none of the data were directly relevant to determining risk on an individual, rather than on a familial, basis. As part of this thesis, it was decided to use the data held on the EUROPAC FPC database to produce a mathematical model capable of predicting individual cancer risk within a set time period. This model would have potential utility as an accurate method of risk stratification, in counselling high risk individuals and (after completion of prospective testing and validation) for assisting in recruitment to the screening study.

A collaborative venture was initiated with individuals from the Department of Medical Physics and Clinical Engineering at the Royal Liverpool and Broadgreen University Hospital which used the methods set out in section 2.2.2.2. For the model to be mathematically proven it had to be able to withstand tests of both discrimination and calibration. As stated in section 2.2.2.2, 'discrimination' is the ability of the model to separate the subjects into two groups, (those that developed cancer and those that did not) and was tested using Harrell's C-Index which is an extension of the Area Under the Receiver Operating Characteristic (AUROC) Curve. 'Discrimination' is the primary test and values ≥0.7 are acceptable, ideally being as close to 1 as possible[310, 311].

The second method used to test the model was 'calibration'. This is the degree of correspondence between the probability of an event being predicted by the model and the probability of that event having actually occurred. It is tested using Hosmer-Lemeshow analysis with the results quantified as a chi-square ($\chi 2$). Calibration is acceptable if the p value is ≥ 0.05 and it should ideally be as close to 1 as possible[312, 320]. The model was shown to have satisfactory discrimination and calibration for individuals aged from 45-69 years. Discrimination was lost (i.e. was <0.7) outside of this age range due to the low number of pancreatic cancers in those age groups. A sample of model output from the WinBUGS software is shown in figure 16 for individuals aged 45-49 years, with all discrimination and calibration outputs summarised in the subsequent table, table 7.

Figure 16: Model Output for the Age Range 45-49 Years

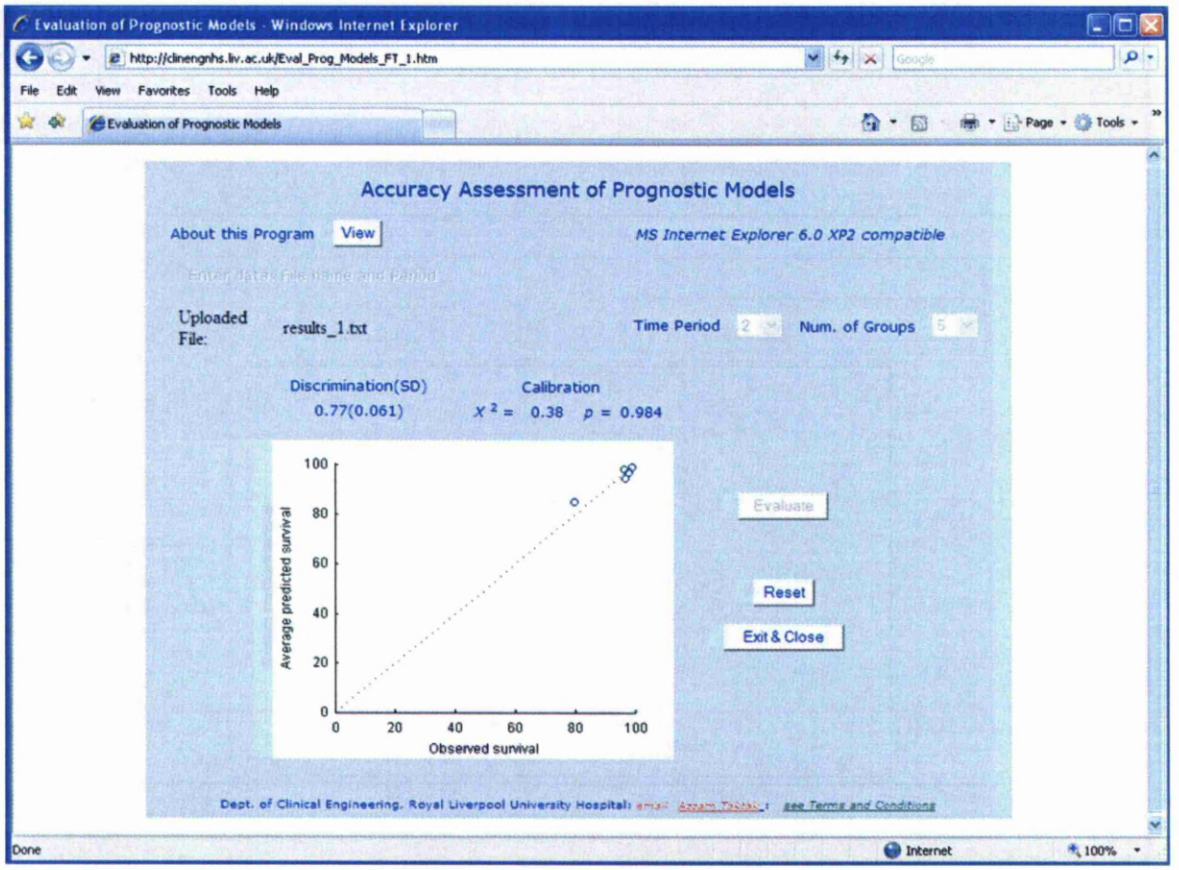


Figure showing the model output for high risk individuals from FPC kindreds aged 45-49 years. Discrimination is shown to be 0.77, with the χ^2 value used to measure model calibration being 0.38, with a p value of 0.984. Discrimination is the primary test and values ≥0.7 are acceptable, ideally being as close to 1 as possible. Calibration is acceptable if the p value is ≥0.05 and should ideally be as close to 1 as possible.

Table 7: Summary of Discrimination and Calibration of the Mathematical Model

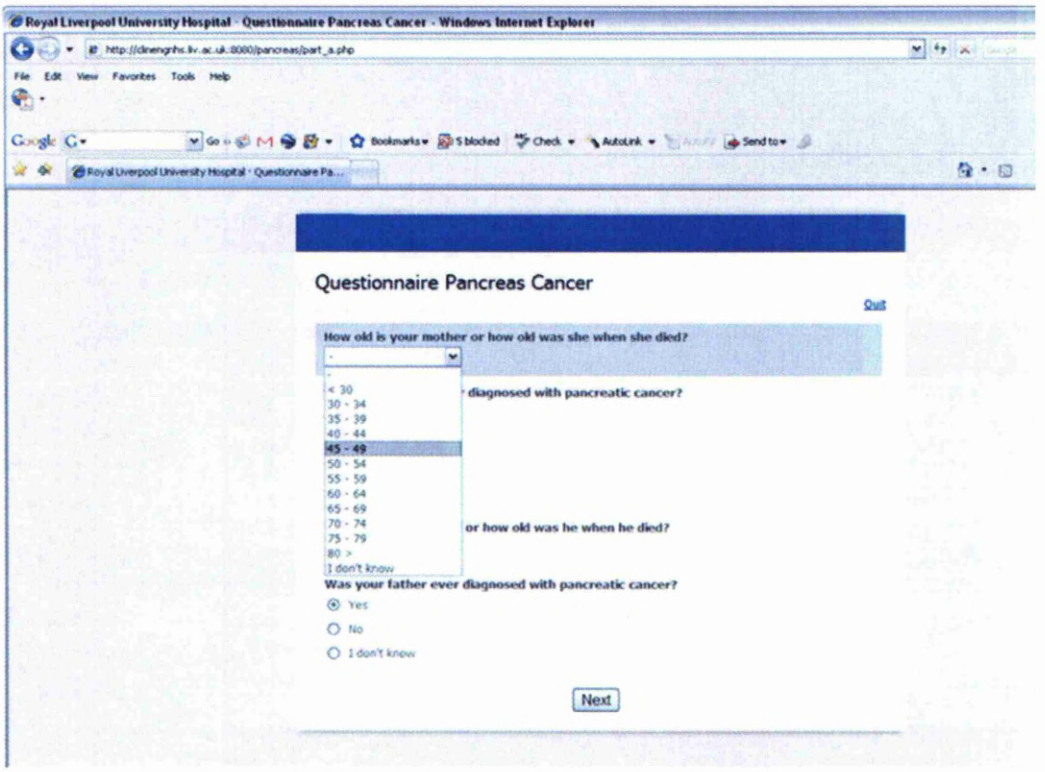
Table summarising the values determined for discrimination and calibration for each group assessed. Discrimination is the primary test and values ≥0.7 are acceptable, ideally being as close to 1 as possible. Calibration is acceptable if the *p* value is ≥0.05 and should ideally be as close to 1 as possible. Discriminatory power was lost in the young and old due to the low number of cancers in these groups. Calibration was adequate until the age of 79 years, but in the absence of acceptable discrimination, the model is only valid for those aged 45-69 years.

Age in Years	Total Cancer Cases	Discrimination (Standard Deviation)	Calibration χ^2	Calibration P value
40-44	21	0.66 (0.120)	0.02	1.0
45-49	33	0.77 (0.061)	0.38	0.984
50-54	56	0.75 (0.041)	0.05	1.0
55-59	77	0.72 (0.036)	0.83	0.935
60-64	120	0.72 (0.035)	1.72	0.787
65-69	88	0.72 (0.033)	5.78	0.216

The Online Interface

The computer interface that was developed permitted both data entry and the production of a survival curve online by registered users. The inputted data permitted a decision on which side of the family carried the presumed FPC gene and data were gathered as fully but efficiently from that side of the family as possible. At the end of the online data collection process, entered data were used to produce a summary sentence. If this summary sentence was accepted as correct, a survival curve was produced using these data which were then stored on the secure server as an excel spreadsheet. During the testing phase, it took approximately ten minutes to enter the data for a family and produce a survival curve, although a new user would likely require slightly longer than this until they were familiar with the system. An example screen from the interface is shown on the following page as figure 17.

Figure 17: Sample Screen from the Online Interface



An example of the online interface. Data entry is organised using either a drop-down or radio button format to make it as user friendly as possible and to ensure that inputted data are in a format that can be used as inputs to the mathematical model.

Pseudo-prospective Testing

The pseudo-prospective testing described in the Materials and Methods investigated whether there was a potential clinical application. The data for 138 individuals were entered, of which 27 subsequently developed pancreatic cancer. The model was able to discriminate between the cancer and the non-cancer cases (C-Index 0.77). Using cut-offs determined by ROC analysis, (a cancer risk in the next five year period of ≥2.84% combined with a Family Index of 0.3), the model indicated that 115/138 should be screened. All 27 cancer cases fell within the screened group with none missed. Put another way, if the model had been used in this group to decide whether to screen them or not, it would have spared one in six individuals the risks of screening for no disadvantage. The results of the calculations performed in *Medcalc* are shown in Table 8. Fisher's Exact test gave a *P* value of 0.0075, but as the aim was to further sub-stratify risk on an individual level, the numbers that would not have to undergo screening are more significant than the statistical result.

Table 8: MedCalc Output Showing Results of Pseudo-prospective Testing

The results of the pseudo-prospective testing. Pedigrees were backdated in 2007 to the year 2000 to see if the model could differentiate between individuals that developed pancreatic cancer and those that did not (between 2000 and 2007). Once the data had been inputted for 138 high risk individuals, cut off points of cancer risk over the next five years of ≥2.84%, combined with a Family Index of 0.3 were adopted to determine who should be screened. The model indicated that screening should be initiated in 88 out of 111 of the ultimately unaffected (no cancer) individuals and in all 27 of the ultimately affected (cancer) individuals. The figures in parentheses are the expected values if the same proportion of individuals had been screened at random, which would have resulted in approximately 5 missed cancers.

	Not Screened	Screened	Totals
No Cancer	23 (18.5)	88 (92.5)	111
Cancer	0 (4.5)	27 (22.5)	27
	23	115	138

3.2.2.3 Serum Glucose

As described in the Introduction diabetes mellitus and failure of glucose regulation in general are closely related to development of pancreatic cancer and possibly risk of pancreatic cancer. In this thesis I will outline the first set of data relating to glucose level measurements as part of a screening program. I will also describe further confirmatory work on glucose levels in newly diagnosed pancreatic cancer patients. The serum fasting glucose data gathered from high risk individuals were from members of both FPC and HP kindreds. Some of these were participants in the secondary screening study, others replied to a written approach. I also collected random glucose data for 282 individuals that had had resections at the RLUH for sporadic peri-pancreatic cancer. It is obviously difficult to compare random glucose samples from one cohort with fasting samples from another. An exact relationship between random and fasting glucose has not been proven, although for the purpose of diagnosis of diabetes, fasting values of ≥7 mmol/L and random (or post-prandial) values of ≥11.1 mmol/L were laid down by the World Health Organisation (WHO) in 1999[321].

The glucose data from EUROPAC's screening studies require further maturation before they can be properly analysed. In the absence of detected abnormalities leading to surgery in the FPC group, there is no way of looking at fasting glucose levels in either those with PanINs or early cancer. Analysis is also hindered in the HP group by the high incidence of endocrine pancreatic failure, the high number of abnormalities on imaging and the minimal number of proven pre-malignant or

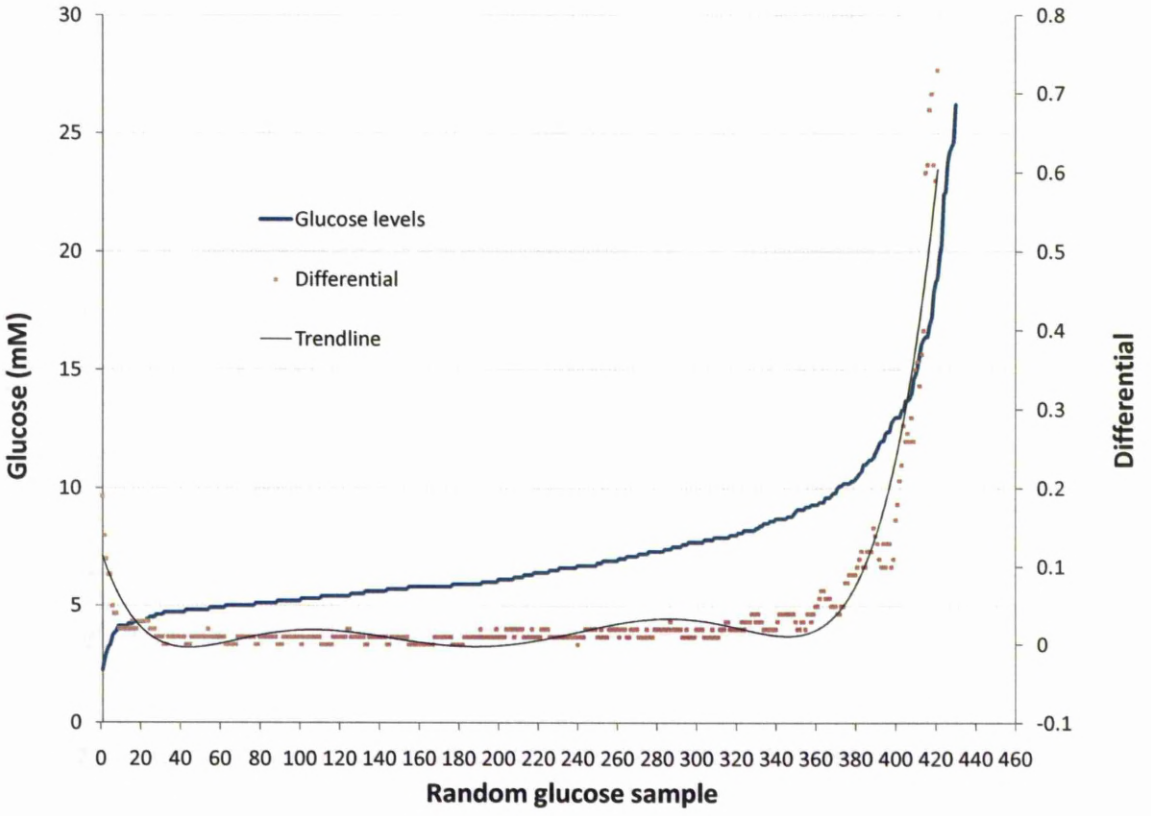
malignant lesions detected. Data collection is continuing and productive analysis should become possible in the future, nevertheless, there are some suggestive data which can be reported here as an initiating point for discussion.

Data for 282 cases of peri-pancreatic cancer were gathered prospectively by members of the research group pre and post surgery over a number of years, but unfortunately glucose data were not included in this before my time with the group. The glucose data for these patients were therefore obtained retrospectively from clinical notes and other records. The pre-operative values were obtained as close to the date of surgery as possible, if possible on day of surgery when it was assumed that these would be fasting samples. The post-operative values were taken as close to four weeks post-surgery as possible. This was an arbitrarily selected time point which was assumed to give the individual time to recover from their surgery. The nature of the data collection and previous hospital computer systems meant that I had to largely rely upon random glucose measurements with little or no contextual details attached to the values. For example, many of the clinical notes had since been destroyed making it impossible to determine in some cases who was known to be diabetic and taking anti-glycaemic medication at the time the blood was taken.

Figure 18 shows the distribution of random glucose levels, clearly demonstrating a population with very low levels (below 5 mM, note the steep gradient) a large population with moderate glucose levels (between 5 and 10mM, note the almost flat curve) and a population with very high glucose (above 10mM glucose, note the steep gradient). The steep increase in gradient started between 10 and 11 mmol/L so the

WHO value of 11.1 mmol/L[321] was accepted as indicative of diabetes for the purposes of the analysis in the rest of this section,

Figure 18: Distribution of Random Glucose Values in Sporadic Peri-pancreatic Cancer



A graphical representation of the number and distribution of 430 ranked pre-operative random glucose measurements (shown in blue) taken from 282 individuals that had resections at the Royal Liverpool University Hospital for sporadic peri-pancreatic cancer. The ranked number is shown on the *x* axis (lowest rank =1, highest ranked =430), with the glucose level shown on the *y* axis. The differential is shown in red, with the trend line for the differential shown in black. Given a normal distribution (i.e. random variation around a mean), the ranked values should increase gradually and evenly. That would mean that the differential of the curve (change in value per ranked sample) should be constant and the line should be horizontal. The trendline for the differential in this figure shows that we get abnormal values before sample 20 and after sample 380.

The glucose data were grouped by diagnosis (PDAC, ampullary and cholangiocarcinoma) and have been displayed below in table 9. There were no significant differences in age and gender between the diagnostic groups. Chi-squared testing showed no statistically significant differences in terms of gender (p=0.791) and Mann-Whitney-U testing showed no significant difference between median ages in the respective groups (p=0.417).

Table 9: The Demographics of Peri-pancreatic Cancer Patients with Glucose Data.

Table to show the gender and age of peri-pancreatic cancer patients that had random glucose levels available both pre and post surgery. They were analysed together and by cancer type. There were no statistically significant differences between the cancer groups, when analysed for either gender or age.

	Combined	Pancreatic Ductal Adenocarcinoma	Ampullary Adenocarcinoma	Cholangio-carcinoma	P value
Number of patients	282	155	77	50	
Gender (M:F)	155:127	88:67	41:36	26:24	0.791[a]
Median Age in years with (IQR)	66.4 (n=282)	66.5 (66.5-72.4) (n=155)	67.3 (56.0-73.3) (n=77)	65.1 (58.4-70.3) (n=50)	0.417[b]

a= Chi-squared test using 3x2 contingency table

b= Mann-Whitney-U test

One significant finding from examining the retrospective glucose results from those with peri-pancreatic cancer was that hyperglycaemia (serum glucose ≥11.1 mmol/L) was not only more common in the PDAC group compared to those with either ampullary or cholangiocarcinoma, but it also appeared to resolve after surgery in this group. There were 27 patients with PDAC, who had pre-operative hyperglycaemia, a resection for PDAC and a post-operative glucose result. Post operative results were missing for a further two individuals and they were excluded. Of the 27 with all data, 25 individuals had resolution of their pre-operative hyperglycaemia following surgery. Conclusions are weakened by the lack of data on hypoglycaemic agents and diabetic status but it is noteworthy that there was no significant change in pre-operative hyperglycaemia following resection for either ampullary or cholangiocarcinoma. These data are summarised on the following page in table 10.

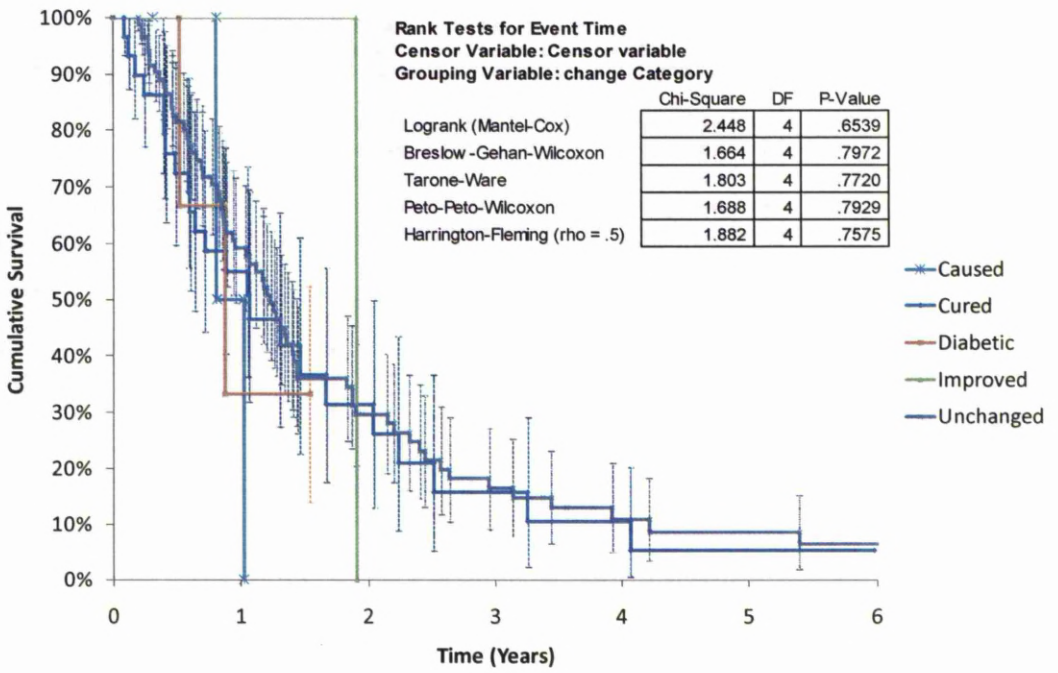
Table 10: Pre- and Post-operative Glucose According to Tumour Group

Table showing the pre- and post-operative random glucose levels dichotomised as either ≤11.0 or ≥11.1 mmol/L by tumour type. The pre-operative values were obtained as close to the date of surgery as possible, if possible on day of surgery (when it was assumed that samples would be fasting). The post-operative values were taken as close to four weeks post-surgery as possible (an arbitrarily selected time point assumed to give individuals time to recover from surgery). Pre-operative diabetes (definition as described in the text based on WHO classifications[321]) was more common in PDAC patients (24%) than in those with either ampullary (9%) or cholangiocarcinomas (14%), with $\chi 2$ testing giving p=0.035. Post-operative glucose results were available for 27/29 individuals that had a resection for PDAC and had pre-operative hyperglycaemia. Of these 27 individuals, 25 had a glucose result ≤11.0 mmol/L following resection of their tumour, with hyperglycaemia remaining in just 3%. There was no significant difference in glycaemic control between tumour groups following surgery, with $\chi 2$ testing giving p=0.507.

	Preoperative glucose			Postoperative glucose		
	≤11.0	≥11.1		≤11.0	≥11.1	
Ampullary	53	5		51	3	
Cholangiocarcinoma	37	6		39	3	
PDAC	93	29		115	4	
			p=0.035			**p=0.507**

The clinical relevance of the strong relationship between pre- and post-operative hyperglycaemia and tumour type is uncertain. When the PDAC group was analysed with respect to the change in random blood glucose after surgery (see figure 19), no difference in survival was shown between the groups irrespective of whether glycaemic control improved, remained unchanged or worsened following resection of the pancreatic ductal adenocarcinoma. There was also no relationship between glucose levels and survival in the other tumour groups (data not shown).

Figure 19: Survival in Those With a Resected Pancreatic Ductal Adenocarcinoma Analysed by Change in Random Glucose Levels

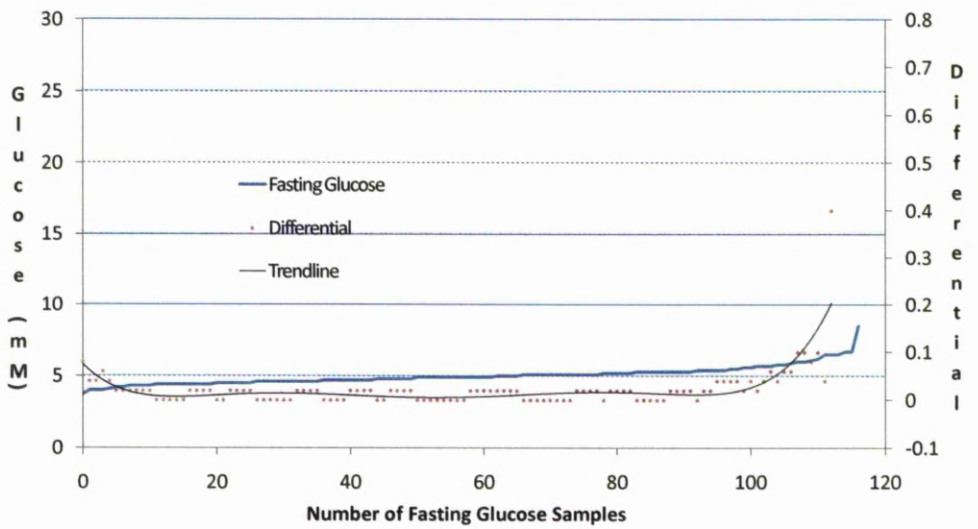


At Risk							
Caused	3	0	0	0	0	0	0
Cured	29	13	6	3	2	1	1
Diabetic	3	1	0	0	0	0	0
Improved	1	1	0	0	0	0	0
Unchanged	83	42	18	10	5	4	3

A Kaplan-Meier survival curve to show the potential differences in survival between those that had a pancreatic resection at the Royal Liverpool University Hospital for PDAC, analysed by the difference between pre-operative and post-operative glucose levels. A cut off of ≥11.1 mmol/L was used as *indicative* of diabetes. The 'caused' group had a glucose <11.1 mmol/L pre-operatively and a level ≥11.1 mmol/L post-operatively. The cured group had a level ≥11.1 mmol/L pre-operatively and a glucose <11.1 mmol/L post-operatively. The diabetic group had a glucose level ≥11.1 mmol/L both pre- and post-operatively, with no marked (>10%) change. In the improved group, the glucose level was ≥11.1 mmol/L both pre- and post-operatively, but there was a marked (>10%) improvement after surgery. The largest group 'unchanged' had a glucose level <11.1 mmol/L both pre- and post-operatively. The most striking feature of this figure is the lack of difference in survival between those patients that had apparent diabetes before surgery (but not after) and the group where there was good glycaemic control pre- and post-surgery.

As described above, the conclusions that can be drawn from analysis of the fasting glucose in high risk individuals are limited as there has only been a single participant with proven PanIN lesions. The distribution of fasting samples for members of EUROPAC FPC kindreds is displayed below in figure 20. The axes have been matched to figure 18, which showed the random glucose results in those having resections for peri-pancreatic cancer, to permit comparison.

Figure 20: Distribution of Fasting Glucose Values in Asymptomatic Members of FPC Kindreds



This figure shows the number and distribution for the fasting glucose samples obtained from members of FPC kindreds. The ranked number of the glucose results is shown on the *x* axis with the glucose level shown on the *y* axis. Glucose levels are shown in blue, with the differential shown in red and the differential trendline in black. None of these individuals are known to have developed pancreatic cancer. Given a normal distribution, the differential of the curve (change in value per ranked sample) should be constant and the line should be horizontal. The trendline for the differential in this figure shows that we get abnormal values before sample 5 and after sample 100.

Although there are no gross abnormalities in the high risk individuals, it is of note that there are some values that are outside of the normal range. These findings, by themselves, do not show that high glucose is a sufficiently early marker to be used in screening, but the results (the lack of any survival difference) in the sporadic cancers suggest that high glucose is not a marker of late disease.

Since the completion of this aspect of my thesis, a number of groups have published regarding the relationship between pre and post operative diabetes on survival from pancreatic cancer. Although results have varied, overall, the data do not support a relationship between survival and diabetes. This is consistent with my own findings and is contrary to my expectations at the outset of my period of research[322, 323].

3.2.3 Risk Stratification in HP

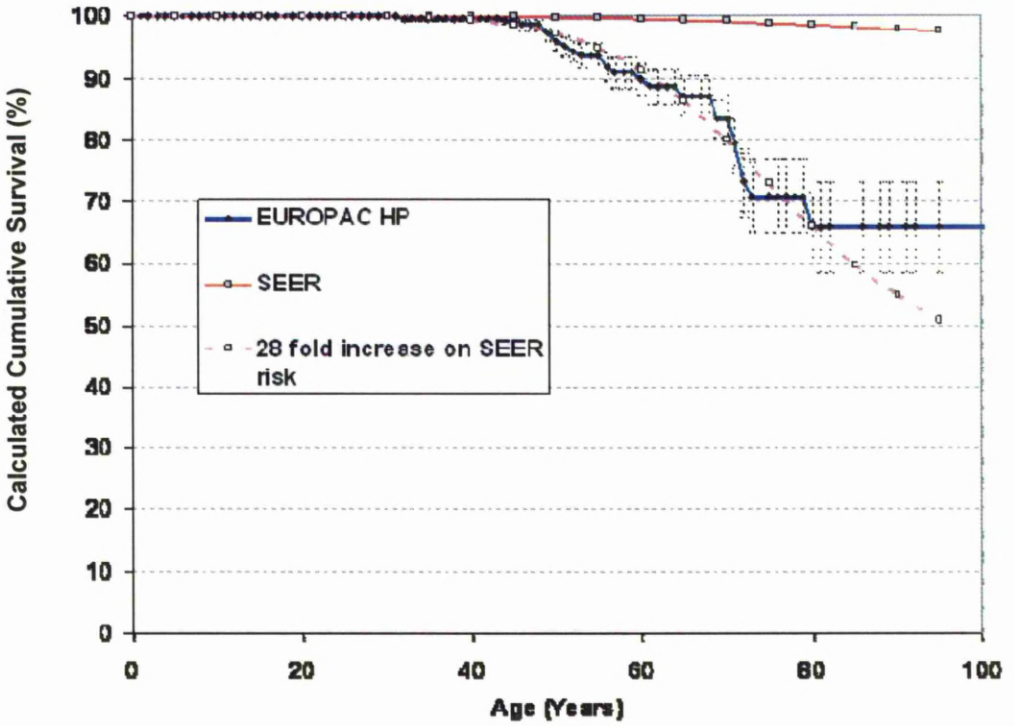
As stated previously, prior to the work that has gone into thesis, the two most common HP mutations had been well characterised, but almost nothing was known about the phenotypes produced by (and the cancer risk caused by) the rarer *PRSS1* mutations, including the third most common *PRSS1* mutation, p.A16V. Before discussing the characterisation achieved in p.A16V kindreds, the cancer risk in all HP kindreds will be described.

3.2.3.1 What is the Cancer Risk in HP Kindreds?

The existence of an increased cancer risk in HP was first identified by Lowenfels *et al* in 1997[91] but was far more accurately characterised by Howes *et al*[92] and then Rebours *et al*[93] using EUROPAC and French data in 2004 and 2008 respectively. These works inevitably concentrated on the most common HP mutations, p.R122H and p.N29I, where statistical analysis was made easier by their higher prevalence.

The lifetime cancer risk in all affected individuals from EUROPAC HP kindreds (irrespective of the causative mutation) has been compared to that of the general US population using the SEER data. This is shown below in figure 21 and the relative risk in affected individuals has been shown to be 28-fold that of the general population.

Figure 21: Comparison of Cancer risk in EUROPAC HP Kindreds and the United States General Population.



At Risk	0	10	20	30	40	50	60	70	80
SEER (millions)	21.8	18.9	15.8	12.9	9.8	6.4	3.7	2.1	0.8
EUROPAC HP	497		121		27		3		

Figure to illustrate the relative risk of pancreatic cancer in affected individuals from HP kindreds compared to that of the United States general population. The cancer risk in individuals affected by HP approximates to a 28-fold increased risk over members of the general population of the United States (SEER). The x axis shows age of individuals in years. The y axis shows calculated cumulative survival shown as a percentage. This is derived from calculations of risk at each specific age point. This calculation is required permit comparison between the cross-sectional SEER data and the longitudinal EUROPAC data.

3.2.3.2 Characterising the Phenotype of the p.A16V Mutation of PRSS1

Pancreatic cancer is therefore a significant problem in HP kindreds. Characterisation of the phenotype in p.A16V kindreds (not just the cancer risk described here, but also onset of pancreatitis and pancreatic failure described earlier in this chapter) had always been limited by rarity of the mutation and the associated low numbers. The first challenge was therefore to identify and recruit as many p.A16V kindreds as possible.

Identification of p.A16V in EUROPAC Families

EUROPAC's p.A16V families were recruited because their phenotype suggested an autosomal dominant predisposition; p.A16V was originally reported in idiopathic pancreatitics. Preliminary analysis of existing EUROPAC families suggested that there were differences between p.A16V and the true HP mutations. Collaborators around the world were contacted to gather as many p.A16V families as possible to enable the mutation to be more accurately characterised. A total of 10 kindreds were identified. Six had a phenotype consistent with HP, three with idiopathic disease and one with FIP. Altogether 37 mutation carriers were identified although only 22 of these were clinically affected by pancreatitis. There were a total of three pancreatic cancer cases although only one had definitely been affected by pancreatitis. Data were also collected for age of onset of pancreatitis and numbers affected by endocrine and exocrine pancreatic failure. The data relating to pancreatitis and its

non-cancer outcomes have been shown above as survival curves but are also summarised in table 11 to put them in the context of cancer risk.

Table 11: Summary of EUROPAC p.A16V Families, January 2009

Each p.A16V family is described including the number of individuals with symptomatic pancreatitis. The median age of onset of pancreatitis is derived using the method of Kaplan-Meier. Median age of onset is given for all patients with pancreatitis or for all patients with p.A16V mutations (censoring at age of last contact for unaffected individuals). IQR is the age of 25% incidence to the age of 75% incidence. The numbers of individuals diagnosed with diabetes, malabsorption or pancreatic cancer are shown in the final three columns. Absolute totals are in plain text, with totals in those affected by pancreatitis in parentheses.

Table 11: A Summary of EUROPAC p.A16V families, January 2009

Family	Classification	Number testing positive for p.A16V* (with pancreatitis)	Median age of onset (IQR) All patients testing positive for p.A16V*	Median age of onset (IQR) Patients with pancreatitis	Endocrine pancreatic failure (with pancreatitis)	Exocrine pancreatic failure (with pancreatitis)	Pancreatic cancer cases (with pancreatitis)
A	HP	7(3)	18 years (7-53)	10 years (5-21)	4(0)†	1(1)	0
B	HP	2(2)			2(1)	2(2)	0
C	HP	2(2)			0	0	0
D	HP	4(4)			0	0	0
E	HP	5(2)			0	0	1(0)
F	HP	3(2)			2(2)	2(2)	1(0)
G	Idiopathic	3(1)	Not reached (5- Not reached)	2 (1-5)	1(0)†	0	0
H	Idiopathic	3(1)			1(1)	1(1)	0
I	Idiopathic	4(1)			0	0	0
J	Single Generation	4(4)	27 (25-28)	27 (25-28)	0	2(2)	1(1)
Totals		37(22)	26 (7-54)	10 (5-26)	10(4)	8(8)	3(1)

*Including deduced carriers; †Including one case confirmed not to have the p.A16V mutation

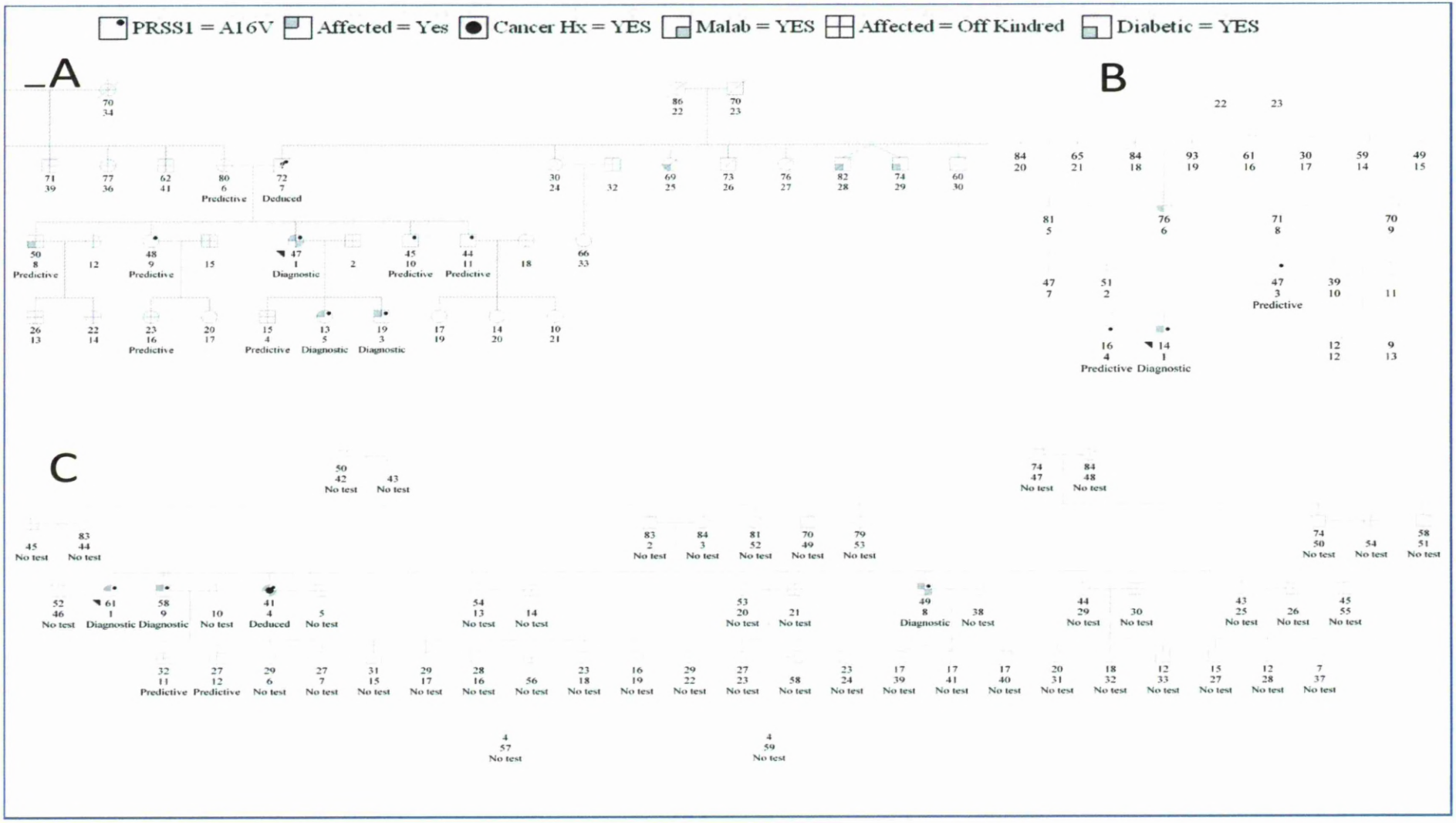
The Variable Phenotype in p.A16V Families

As shown in the previous table, the p.A16V mutation of *PRSS1* is not exclusively a HP mutation. Sample families from each group are shown on the next page in Figure 22.

Figure 23: Sample Pedigrees Indicating the Variable Phenotype of p.A16V

Ages and unique personal identification numbers are shown below each family member followed by a description of the type of p.A16V test carried out. Arrows indicate index cases. A shaded left upper quadrant indicates that the individual is affected by pancreatitis, shading of the left lower quadrant indicates diabetes mellitus, with a shaded right lower quadrant indicating exocrine pancreatic failure. The presence of a dot in the right upper quadrant signifies the presence of a p.A16V mutation, individuals assumed not to have a p.A16V mutation are described as off kindred and are marked with a cross. The central black circle indicates the presence of a confirmed pancreatic adenocarcinoma. A) Displays autosomal dominant inheritance. B) Includes an idiopathic case of pancreatitis. C) Defined as having familial idiopathic pancreatitis; as cases of pancreatitis, diabetes and pancreatic cancer are restricted to a single generation.

Figure 22: Sample Pedigrees Indicating the Variable Phenotype of p.A16V



Family A is an example of a kindred described as having HP (Figure 22A); as with all such families the definition of HP was based on multiple cases and preceded genetic testing. All families in this group would be defined as HP regardless of whether mutations were detected. In this case the family was identified after the proband's children (individuals 3 and 5) were affected by pancreatitis. Both p.R122H and p.N29I were excluded and a p.A16V mutation in one copy of *PRSS1* was detected in the proband (individual 1).

It remains possible that p.A16V is an associative rather than a causative mutation, but testing has yet to identify an affected individual that does not carry p.A16V in any of the p.A16V HP kindreds and mutations in other candidate modifier genes (*CFTR* and *PSTI*) have not been detected. 'Affected' means that individuals have reported symptoms of pancreatitis and the possibility of sub-clinical disease cannot be excluded in other family members.

In Family A one of the proband's siblings has diabetes (individual 8). This could be a manifestation of pancreatic inflammation in the absence of pain, but this individual does not carry p.A16V. It is possible that, at least in this kindred, p.A16V is modifying the symptoms of an underlying inherited pancreatic disease, with the mutation increasing the chance of pain. Diabetes mellitus is, however, not uncommon, and the cases in such families may well be coincidental.

The p.A16V mutation could explain the pancreatitis in all the p.A16V HP kindreds, but even in these families, not all carriers had explicit symptoms. Unavoidably, most unaffected individuals in the families were not tested. Of the 23 individuals who were

confirmed to have the p.A16V mutation only 15 had reported symptoms of pancreatitis. All of the proband's siblings in Family A were tested for p.A16V, three (individuals 9 to 11) carried the mutation but none reported symptoms. The proband's mother was tested and no mutation was detected, indicating that the mutation was inherited from the father (individual 7). There were no cases of pancreatitis present within any of his eight siblings, although three were affected by diabetes mellitus. No testing was conducted within this generation. Two of these diabetics were identical twins, consistent with a genetic predisposition to diabetes in this part of the kindred. It is, however, important to note that if the risk of diabetes is independent of the p.A16V mutation and the inherited risk accounts for the diabetes seen in individual 8; then the father of the proband (individual 7) must have carried both the p.A16V mutation and the predisposition to diabetes without developing symptoms.

At the time of guillotine for the p.A16V paper (January 2009) the EUROPAC database contained 142 families with a phenotype consistent with idiopathic disease that had undergone genetic testing. Three of these have a proven p.A16V mutation (2.1%). The families are named G (Figure 22B), H and I (family trees not shown). In all cases referral was atypical for EUROPAC, in that there was no family history. Unlike any of the HP families, genetic testing was performed before recruitment to the study. For example, Family G (Figure 22B), was referred after an 11 year old boy (individual 1) was admitted to hospital with recurrent abdominal pain which had first started at the age of 5. An appendicectomy was performed, histology showed no evidence of inflammation. He subsequently developed a pancreato-pleural fistula

requiring a chest drain. Due to his personal history, his clinicians requested testing for a *PRSS1* mutation. Subsequent to discovery of a p.A16V mutation, his mother and brother (individuals 3 and 4) underwent testing and have been confirmed as unaffected p.A16V carriers. Neither of the proband's maternal grandparents (individuals 8 and 9) are affected and testing has not been performed in either case. Similarly in families H and I there was childhood onset disease, consistent with the original identification of p.A16V in sporadic cases identified in paediatric units[191]. This may represent referral bias rather than a feature of p.A16V *per se.*

At the time of guillotine for the p.A16V paper there were 40 pancreatitis families on the registry, where despite thorough investigation, the disease appears to be limited to a single generation with no evidence for spontaneous mutation. This phenotype is consistent with a multigene or recessive syndrome. One of these 40 families, Family J (Figure 22C), carries a p.A16V mutation (2.5%). It was referred after pancreatitis was diagnosed in four of eight siblings, one of whom had already died from a histologically proven adenocarcinoma of the pancreas at the age of 44. The other affected siblings all tested positive for the p.A16V mutation. The remaining four siblings were unaffected and declined genetic testing. The pancreatic cancer case was not tested for p.A16V although she had been affected by pancreatitis, prior to developing malignancy. Both the parents and offspring were asymptomatic and testing was not performed in either the preceding or subsequent generation.

Phenotype of p.A16V Compared to Other *PRSS1* Mutations

A visual comparison of pedigrees indicates that there are differences between the phenotype of p.A16V and the phenotype associated with other *PRSS1* mutations. However, the low incidence of p.A16V and the even smaller numbers of carriers affected by diabetes mellitus, malabsorption or pancreatic cancer means that there is insufficient power for a meaningful statistical analysis. Nevertheless, the data summarised in table 11 confirm that cancer and both endocrine and exocrine failure are features that occur within p.A16V families.

Figures 8-10 in section 3.1.2.3. do not provide adequate evidence to suggest a difference in disease progression between p.A16V and the other *PRSS1* mutations, but as described above, there is a clear difference in penetrance. With just three cancer cases there is no possibility of proving a difference between age of onset of cancer in p.A16V and any other group (as Kaplan-Meier analysis of only affected individuals would lack adequate power) but the unavoidable impression is that p.A16V carriers have a lower cancer risk than carriers from the other mutation groups, if for no other reason than penetrance being lower. Cancer risk in p.A16V may equate to that seen in individuals with non-genetic chronic pancreatitis, but again, the low numbers prohibit meaningful statistical analysis.

3.3 Secondary Screening

As stated previously, secondary screening of high risk individuals for early pancreatic cancer had been happening on a piecemeal basis under local ethical approvals for several years. The second aim of this thesis was to pilot a trial of secondary screening in high risk individuals. Having planned the study, the next step was to obtain the necessary ethical approvals.

3.3.1 Ethics

As stated in section 2.3.2, I made two separate National Research Ethics Service (NRES) applications for the *EUROPAC Study of Secondary Screening for Early Pancreatic Cancer* in the FPC and HP groups respectively. The FPC application was granted by Warwickshire Research Ethics Committee (REC Reference 07/H1211/96) with the HP application being approved by the Central Manchester Research Ethics Committee (REC Reference 07/H1008/153). Samples of the patient information sheet and consent form from the FPC application have been included in chapter 6 of this thesis as an appendix. The main concern of the ethics committee was the issue of gender discrimination. This contrasted with the concerns of the research group which will be discussed in Chapter 4.

3.3.2 Secondary Screening in High Risk Kindreds (June 2008)

The results in this section were prepared for a presentation given to the EUROPAC study group in late June 2008 and had been collated over the previous 2 months. Many of the data had been collated prospectively but they were checked and any gaps in the data were closed. The data for secondary screening in FPC kindreds will be presented first followed by those for the HP kindreds.

3.3.2.1 Secondary Screening in FPC (June 2008)

Taking 30th June 2008 as the date of guillotine for these data, a total of 70 individuals from FPC kindreds had entered the EUROPAC study of secondary screening for early pancreatic cancer in high risk groups. Of those, 65 remained within the study and wished to continue with further investigations. The reasons for the loss of the five individuals that had entered the screening study and then left it were mixed. One decided to leave the registry entirely, whilst a second stayed on the registry but declined any further investigations. The third patient remained on the registry but emigrated to a country with no collaborating clinicians, whilst a fourth was lost to follow up despite efforts to relocate him. The final individual was withdrawn from the study after additional primary screening and the recruitment of other family members indicated that at least one of the cancer cases in the family was highly unlikely to be pancreatic.

Screening was carried out at seven screening centres within the UK, but the vast majority of investigations were performed at either Liverpool, or the second largest

centre, University College London. Of the 65 individuals that remained within the screening study, 60 were compliant with the protocol, meaning they had had either their baseline investigations within the last three years (if juice analysis then indicated they should follow the standard surveillance pathway) or the most relevant investigation (normally EUS) within the last twelve months if they were following the close surveillance pathway. One of the major challenges was to standardise the management of those that had previously had investigations under local ethical agreements at various centres. The first step was informed consent followed by serum blood samples. These had often not been collected outside Liverpool prior to the national ethics application, but existing results were chased and additional blood forms sent out to individuals not compliant with the protocol. This meant that at time of guillotine for the data displayed in tables 12 and 13, just 15 of the 65 being screened had signed and returned the new consent form; although written consent was in place for everybody under local agreements.

Variation between screening centres also meant that there was no set protocol for blood tests being gathered under local consents. There were just 16 fasting glucose samples available (all collected since the national ethics approval), but a total of 81 CA19-9 results recorded, most of which had been collected using local research ethics committee approvals. Of these 81 CA19-9 samples, five individuals had serum CA19-9 levels greater than the normal range of <35 kU/L. Four of these individuals had levels between 36-44 kU/L. These levels did not correspond to identifiable lesions of concern on imaging. One individual subsequently developed a pancreatic cyst in 2010 which is being managed conservatively. One individual screened at a

satellite centre (under local ethical consent) had a CA19-9 result of 1726 kU/L in November 2006 and 6704 kU/L in June 2007. However, these grossly abnormal CA19-9 results did not correspond to identifiable changes on imaging. Two EUS examinations had been performed (in 2005 and 2007), which were unremarkable. Two CT scans were performed, one in 2005 and a second in November 2006. There was also a MRCP in January 2006, which was also reported normal. She was seen in the clinic in June 2007 when her main problem was a change in bowel habit to towards constipation and a colonoscopy was requested. She was subsequently diagnosed with carcinomatosis with an unknown primary and died in 2008. A biopsy confirmed adenocarcinoma and it is certainly possible, but not definitively proven, that the primary site of the malignancy was in the pancreas.

A total of 64 CT scans had been performed. Eight abnormalities had been detected but these were essentially all incidental findings with none indicating a pancreatic tumour. A total of 73 EUS scans were performed. These detected a total of seven abnormalities. Five were definite benign disease. There were four diagnoses of incidental gallstones and one of chronic pancreatitis. There were two potentially significant abnormalities detected. One was for a pancreatic cyst and the second was pancreatic duct dilatation. Both were discussed at MDT and monitored by interval imaging, with no indication to proceed to a resection. A total of 12 MRI or MRCP scans had been performed. These showed three abnormalities, two were IPMNs which were being managed with interval surveillance and there was an incidental diagnosis of gallstones in an individual screened in London.

Finally, a total of 16 ERCPs had been performed in 16 different participants in the FPC group. These were all carried out at Liverpool. Juice was collected successfully in 11 individuals. Six individuals (of the 16) had their procedure complicated by acute pancreatitis (three cases were where juice was successfully collected and three were in individuals where it was not), giving a rate of 37.5% for all procedures. Of these, as described in the section on adverse events, all settled with conservative management although one individual had sterile pancreatic necrosis and subsequently developed exocrine pancreatic failure requiring supplementation with porcine pancreatic enzymes.

The analysis of the 11 pancreatic juice samples successfully collected showed that seven of the samples had no *K-RAS2* mutations. One individual had a total of four mutations out of the six tested for, with the other three individuals having a single Kras mutation each. *CDKN2A* methylation and *Tp53* results were available for a total of eight individuals. Two individuals had a *CDKN2A* promoter methylation >12% (including the individual with multiple *K-RAS2* mutations), but there were no *Tp53* mutations detected.

In terms of overall clinical impact, negative results meant that two patients entered the 'standard' surveillance pathway and were imaged on a three yearly basis. Conversely, two of the individuals with mutations entered the 'close', rather than the 'standard' pathway.

On the next two pages the secondary screening data for the FPC kindreds will be summarised. Table 12 will show the numbers of individuals screened, the site,

compliance and number of 'screening years'. Table 13 will summarise the numbers of each investigation performed and the number of abnormalities detected.

Table 12: Summary of FPC Screening (June 2008)

Table summarising the numbers of high risk individuals from FPC kindreds that have been screened by EUROPAC collaborators up to June 2008. Numbers (and percentages where applicable) are given for the total that have ever been screened and who are still being screened, these are divided by the relevant screening centre. The number of individuals compliant with the protocol are also given, taken as the most relevant investigation performed in the past 12 months if on the close surveillance pathway, or within the past three years if on standard surveillance. Finally there is a figure given for the total number of 'screening years' in these 65 individuals.

	Number	Percentage
Total Ever Screened	70	N/A
Total Still screened	65	93%
Centre	(n=65)	
Liverpool	35	54%
London	23	35%
Other	7	11%
Compliant with protocol	60/65	92%
Screening Years	133	N/A

Table 13: Summary of Investigations performed in FPC cohort (June 2008)

Table summarising the numbers of investigations performed in high risk individuals from FPC kindreds by EUROPAC collaborators up to June 2008. Investigations are grouped into blood investigations, imaging and ERCPs for collection of pancreatic juice. Abnormal blood results were those outside of the normal reference range. The total number of abnormal imaging results (benign and suspicious) are listed for each modality with numbers of suspicious lesions detected shown in the final column. EUS detected a cyst in one individual and pancreatic duct dilatation in another. MR detected IPMN lesions in two separate individuals. The abnormal result recorded for ERCP relates to the imaging appearances. As stated elsewhere, ERCP was predominantly performed for juice analysis. This detected Kras mutations in four individuals (out of 11 samples), one of whom had multiple *K-RAS2* mutations. Juice analysis results were termed suspicious if ≥2 *K-RAS2* mutations; *CDKN2A* hypermethylation; or *Tp53* mutations were present. The total number of abnormal and suspicious lesions from juice analysis is one less than the sum of the values in that column. This is explained by the individual with the multiple *K-RAS2* mutations also having *CDKN2A* hypermethylation >12%.

	Total Number	Total Abnormal	Suspicious lesions
Blood			
Fasting Glucose	16	0	N/A
CA19-9	81	5	N/A
Imaging			
EUS	73	7	2
CT	64	8	0
MR	12	3	2
ERCP	16	5	2
K-RAS2	11	4	1
CDKN2A	8	2	2
Tp53	8	0	0

3.3.2.2 Secondary Screening in HP (June 2008)

By 30th June 2008, the numbers that had entered the screening study from HP kindreds was lower than in the FPC kindreds. A total of 24 high risk individuals from HP kindreds had had screening investigations, under either local or national ethical agreements at some point. Of those, again, five were no longer being screened. Three of these had been withdrawn from screening as they had been operated on. Two of these were the cases A and B described in detail in section 3.3.3 of this thesis. The third had a total pancreatectomy performed at another centre by a collaborating clinician for intractable pain, rather than on the basis of screening investigations. Of the remaining two HP individuals, one had their investigations suspended by the research team (as screening had been started before the age of 40 by a collaborator under local ethical authority at another centre). The intention is to restart the screening cycle when the individual reaches the age of forty years. The final participant was, unfortunately, lost to follow up.

Screening was located at seven screening centres across the UK, but as with the FPC cohort, the vast majority of individuals were being screened either in Liverpool (n=9), or the second largest centre, University College London (n=4). The remaining six individuals were screened across five other sites, as close to their home address as possible. Of the 19 individuals that remained within the screening study, 18 were compliant with the protocol, meaning as in the FPC cohort, that they had either had their baseline investigations within the last three years (if juice analysis indicated they should follow the standard surveillance pathway) or the most relevant

investigation (normally CT) within the last twelve months if they were under 'close surveillance'. The same problems regarding variation in practice, due to local ethical agreements were encountered as in the FPC cohort. This led to comparatively few blood samples having been collected outside Liverpool before the national ethics application.

At time of guillotine in the HP cohort, just three fasting glucose samples were available and there were 26 CA19-9 results. The fasting glucose results were all within the normal range. The CA19-9 results were more interesting, with two results (in the same individual) outside the normal range of <35 kU/L. There was a result of 137 kU/L and 89 kU/L from 2005 and 2008 respectively. CT imaging throughout this period showed severe chronic pancreatitis with no formed tumour. An ERCP was performed in April 2006. Juice analysis detected no abnormality. At time of writing the individual remains well. She has minimal pain but both endocrine and exocrine pancreatic failure.

A total of 28 CT scans, 31 EUS scans and two MR scans had been performed. One of the problems with imaging in the HP group was that by definition, very few of the scans were normal (see table 15), making detecting early changes of possible emerging malignancy problematic.

A total of 16 ERCPs were performed in the HP group in 11 individuals. There were again some problems obtaining pancreatic juice with samples ultimately being successfully collected at 13 procedures performed in nine individuals (with a total of three failed juice collections in two different individuals). Juice analysis in the HP

subgroup detected 'later' changes than were seen with FPC individuals. Of the 13 samples collected from nine individuals, all were tested for mutations of *K-RAS2.* Five separate individuals had no detectable mutation. There were 7 *K-RAS2* abnormalities detected in four separate individuals. Only one of these was a single mutation and this was in a retest for an individual that had previously had multiple *K-RAS2* mutations. Arginine was the most common mutation detected (present in six samples taken from four separate individuals), with no Aspartate or Alanine mutations detected. There were *CDKN2A* promoter methylation results from 12 samples in eight separate individuals, with four samples in three separate individuals showing *CDKN2A* hypermethylation >12%. *Tp53* mutation testing had been performed eight times in seven separate individuals. There had been a *D245G* mutation detected, although the retest showed no detectable *Tp53* abnormality. This individual is described in this thesis as Case A.

There were no confirmed cases of post-ERCP pancreatitis. One individual had post-ERCP abdominal pain and was observed overnight, but her serum amylase remained within the normal range and she did not require any further medical treatment.

The clinical impact of ERCP and juice analysis was again limited. Of the 19 individuals that remain within the screening study (those operated are excluded from this analysis), two were placed on the close surveillance pathway on the basis of their pancreatic juice analysis results, with one remaining on the standard

surveillance pathway, as their pancreatic juice analysis showed no detectable abnormality.

On the next two pages the above data will be summarised in two tables, which are similar to those displaying the secondary screening data for the FPC cohort in the previous section. Table 14 will show the numbers of individuals screened, the site, compliance and number of 'screening years'. Table 15 will summarise the numbers of each investigation performed and the number of abnormalities detected.

Table 14: Summary of HP Screening: (June 2008)

Table summarising the numbers of high risk individuals from HP kindreds that have been screened by EUROPAC collaborators up to June 2008. Numbers (and percentages where applicable) are given for the total that have ever been screened, are still being screened and sub-divided by screening centre. The number compliant with the protocol (taken as the most relevant investigation performed in the past 12 months if on the close surveillance pathway, or within the past three years if on standard surveillance) is also shown followed by the total number of screening years in these 21 individuals.

	Number	Percentage
Total Ever Screened	24	N/A
Total Still screened	19	79%
Centre	(n=19)	
Liverpool	9	47%
London	4	21%
Other	6	32%
Compliant with protocol	18/19	95%
Screening Years	57	N/A

Table 15: Summary of Investigations Performed in the HP Cohort (June 2008)

A summary of the investigations performed in high risk individuals from HP kindreds by EUROPAC collaborators up to June 2008. Investigations are grouped into blood investigations, imaging and ERCPs for collection of pancreatic juice. Blood investigations were termed as abnormal if they were out of the normal reference range. Imaging investigations were termed abnormal if they showed changes consistent with chronic pancreatitis or other benign disease (including small cysts and pancreatic duct dilatation), with one CT scan showing changes indicative of possible malignancy. In the ERCP section (in contrast to the FPC kindreds), some individuals from the HP kindreds had more than one ERCP. The figures shown are the totals for each group, with data in parentheses being the number of separate individuals. A total of 16 procedures were performed in 11 separate individuals. Juice was collected successfully from 13 procedures in 9 separate individuals, all of which were tested for *K-RAS2* mutations. Twelve samples (from eight individuals) were tested for *CDKN2A* hypermethylation, with eight samples (from seven separate individuals) tested for mutations of the *Tp53* gene. Single mutations of *K-RAS2* were defines as abnormal but not suspicious. Multiple *K-RAS2* mutations, *CDKN2A* hypermethylation (>12%) or those with a Tp53 mutation were termed suspicious. Any disparity between the total with an abnormality or a suspicious lesion and the total of the numbers in that column is explained by the presence of more than one of the abnormalities being present in the same sample.

	Total Number	Total Abnormal	Suspicious lesions
Blood			
Fasting Glucose	3	0	N/A
CA19-9	26	2	N/A
Imaging			
EUS	31	16	0
CT	28	24	1
MR	2	2	0
ERCP	16 (11)	11 (6)	7 (5)
K-RAS2	13 (9)	7 (4)	6 (4)
CDKN2A	12 (8)	4 (3)	4 (3)
Tp53	8 (7)	1 (1)	1 (1)

3.3.3 Management of Detected Abnormalities

In this section the management of abnormalities detected by screening will be discussed. The process for all abnormalities will be described, followed by the specifics on the two members of HP kindreds that had resections under the auspices of the secondary screening study.

3.3.3.1 All Abnormalities

Any significant abnormality identified by the screening process was discussed at the next weekly EUROPAC meeting. If changes were considered significant by the EUROPAC study group, cases were presented for discussion at the next Regional Pancreatic MDT meeting, where any clinical decisions were taken. The process has been described in section 2.3 and summarised in figure 4. At the commencement of my research period when all screening was being performed under local consents, the return of results to the EUROPAC was dependent on local screening clinicians. Real time tracking of results markedly improved following the introduction of the national MREC ethical agreement, although retrospective audits remained necessary to ensure all results had been collated. During the course of my research, two patients came to resection on the basis of screening results and other symptoms. The third resection performed on a HP individual at another centre for intractable pain will not be described as the screening study had no bearing on the clinical decision making. Both resections performed as a result of the screening study were

undertaken in members of HP kindreds. They will be referred to as cases A and B and are described below.

3.3.3.2 Case A

Case A was a female, born in 1958 and proven to be a p.R122H mutation carrier from a HP kindred. Her mother had died from pancreatic cancer at the age of 61 years. She was initially referred in late 2005 and was seen in January 2006 in the outpatient clinic in Liverpool by the then EUROPAC research fellow, Mr Vitone, and Professor Neoptolemos. The reason for the referral was her interest in the secondary screening study. She gave informed consent, bloods were drawn and a CT and ERCP for juice analysis were requested. The CT showed no pancreatic abnormality but the juice analysis showed her to have multiple Kras (Arginine, Serine and Cysteine) mutations; a p53 (G245D) mutation and raised *CDKN2A* promoter methylation at 18% (the upper limit of normal being 12%). According to the Bayesian analysis performed as part of Yan *et al*[220], these three changes gave an estimated risk of developing pancreatic cancer of about 90%[220]. An EUS was requested and was performed in July 2006. Despite being a proven p.R122H mutation carrier the EUS showed normal pancreatic tissue with no evidence of chronic pancreatitis. The case was discussed at MDT and it was decided to repeat the ERCP and juice analysis. This was performed in August 2006 without complication. The subsequent juice analysis detected Kras mutations (Arginine and Valine); raised *CDKN2A* promoter methylation, which increased to 50-100%, but there was no *Tp53* mutation

detected. A further EUS was performed in November 2006 which detected changes consistent with mild chronic pancreatitis.

Case A came back to the outpatient clinic in March 2007 and was seen by myself and Professor Neoptolemos. Over the winter she had had three attacks of pain with one admission to her local district general hospital with acute pancreatitis with documented hyperamylasaemia. It was decided to repeat her EUS and ERCP in May 2007, which would have been six months since her last EUS and ten months since her last ERCP for juice analysis. A few days after the clinic, she was readmitted locally with acute pancreatitis and again had raised serum amylase. She contacted Professor Neoptolemos and was eventually listed for a spleen and duodenum preserving total pancreatectomy. This was performed in May 2007 and she recovered without complication. Her histology showed normal pancreatic parenchyma with neither malignancy nor PanIN lesions in the blocks examined. I reviewed her post-operatively in the outpatient clinic. She recovered well, though required significant doses of both endocrine and exocrine pancreatic supplements. She had an element of exocrine pancreatic failure before surgery but the insulin dependent diabetes was a result of the operation itself.

3.3.3.3 Case B

Case B was a proven p.N29I mutation carrier born in 1959 from a large HP kindred. She was referred just after case A and was first seen in the outpatient clinic by Professor Neoptolemos in February 2006. She was recruited to the secondary screening study and a CT and ERCP were requested. A clinical diagnosis of malabsorption was made and she was commenced on Creon. The baseline CT showed no abnormality. The ERCP was successful in collecting pancreatic juice. The analysis detected mutations in *Kras* Arginine, Serine, Cysteine and Valine; *CDKN2A* promoter methylation was also raised at 17.6% (<12% being the normal range). Following these results, an EUS was requested. This was performed in November 2006 and showed no significant abnormality.

As the significance of the molecular analysis was unproven on a prospective basis, it was decided to repeat the EUS six months later. This was done in May 2007, when a cystic lesion was detected. A CT of the pancreas was requested, confirming the presence of the lesion, along with widespread calcium deposition, although there was no evidence of metastatic disease. The case was discussed at MDT and the decision was to bring the patient back to clinic to discuss either ongoing close surveillance or resection. She chose to go ahead with resection and was listed for a spleen and duodenum preserving total pancreatectomy. This was performed without complication and the histology showed PanIN 1a and 1b with focal areas of PanIN2, but no malignancy. Post-operatively, she developed endocrine failure, in addition to her pre-existing exocrine pancreatic failure.

3.3.4 Cost Analysis

The secondary screening study has led to the use of significant numbers of investigations and has yet to discover its first cancer. An audit of the secondary screening study was completed in June 2008. The numbers of investigations have been multiplied by the cost per investigation used in the last paper on cost analysis published in the literature[300], with comparisons discussed in section 4.3.6. It should be stressed that these costings are only for the imaging investigations. No attempt has been made to estimate expenditure on other costs such as serum blood investigations, staff and labour costs of either the EUROPAC research fellow or the scientists analysing the pancreatic juice. Furthermore, there is also no allowance made for clinical costs, including the two resections performed as part of this study.

Table 16: Estimate of EUROPAC Expenditure on Secondary Screening Imaging

Table giving estimated costs per imaging investigation (costs taken from Latchford *et al*[300] to permit comparison); the number of each test performed in both FPC and HP kindreds; with total estimated cost of each investigation in the right hand column. Total expenditure on imaging alone was >$113 000 to July 2008.

Test	Estimated Cost per Test ($)	Total number of each test (FPC)	Total number of each test (HP)	Total Cost per test to June 2008.
CT	$268	64	28	$24 656
MR	$268	12	2	$3 752
EUS	$590	73	31	$61360
ERCP	$740	16	16	$23 680
Total				**$113 448**

3.3.5 Adverse Events

The only adverse events that I am aware of or were reported during my period of research were a run of cases of acute pancreatitis triggered by collection of pancreatic juice at ERCP in individuals from the FPC kindreds. There were a cluster of three cases in the Spring and Summer of 2006 that prompted an audit that was one of my first tasks when I started my research.

All ERCPs that had been performed where pancreatic juice had been collected were included. All screening ERCPs were included, whether juice had been successfully collected or not. Many of the ERCPs had been performed in symptomatic individuals who required the procedure as part of their treatment. Juice was collected to test and validate the collection and analysis of pancreatic juice for cancer associated mutations that led to the Yan paper[220] prior to the commencement of the screening studies. Those with benign disease acted as controls. Of a total of 312 ERCPs, 108 were performed for biliary stone disease, 46 were performed in chronic pancreatitics, 44 had pancreatic or biliary tract cancer, with 114 performed for other benign disease (including strictures and research). Of the 312 individuals, thirteen had a serum amylase >450 U/l records on the hospital computer system following the procedure, giving an overall rate of 4.2% (13/312). However, of the thirteen cases, six had taken place within the twelve asymptomatic members of FPC kindreds that had had an ERCP for collection of pancreatic juice for screening purposes at the time the audit was initiated. The pancreatic duct had been cannulated in three cases, but not in the other three. The other seven cases took place in patients with both

gallstones (n=3) and chronic pancreatitis (n=4), the pancreatic duct having been cannulated in one patient from each of these two groups. The overall rate of 4.2% compares well with rates quoted in the literature but the rate of 50% in FPC screening participants at time of guillotine for this audit is obviously a marked anomaly. All cases of pancreatitis audited were mild[324], except for the final case where the individual developed approximately 50% pancreatic necrosis. This did not become infected and he was discharged home without intervention but subsequently required exocrine pancreatic enzyme supplements. All other patients were discharged without further complication. The above audit showed that there was not a single case of post-ERCP pancreatitis in members of the HP kindreds. The issues surrounding this will be discussed in the following chapter.

4 Discussion

4.1 Primary Screening

Primary screening is hugely time consuming and of variable utility. It is possible to fully characterise a family from the data provided by family members and prove the diagnoses using available public records. In other kindreds, even where knowledge of other relatives is equally good, one can hit a problem relatively early in the process that cannot be overcome, with the result that the family remains poorly characterised. When performed effectively, primary screening is often the most important tool available in family characterisation and risk stratification. A shortcoming is that in the FPC kindreds, in the absence of a genetic test, risk is generally determined on a familial rather than individual level. It is the inability to differentiate between the varying risks of family members that has driven attempts to improve characterisation of risk on an individual basis described in this thesis.

4.1.1 How Effective Are We At Identifying High Risk Families?

As stated above, the process of traditional primary screening can prove informative for both study participants and their clinicians. In other cases, it is of minimal benefit and even when thoroughly performed, can cause its own problems with the potential for both false positives and missed families.

The single simplest test for the effectiveness of EUROPAC's primary screening programme is the number of prospective cancers detected in these families either by regular follow up or within the secondary screening study. The pseudo-prospective

testing of the risk stratification model identified 27 prospective cancers in EUROPAC's FPC families between 2000 and 2008, in line with expectation. Given this, it is surprising that we have identified so few cancers in our screening cohort. Part of this could be explained by the fact that the screening period has not been sufficiently long, but one must also consider the quality of families recruited in the past and the entry criteria to the screening study. EUROPAC has withdrawn one individual from both the FPC and HP arms of the screening study (recruited under local ethical approvals) due to them not meeting the inclusion criteria drawn up for the secondary screening study. This illustrates the problem of local centres submitting patients to screening, due to either individual or centre demand, in contradiction of the protocols laid down by the research group.

In contrast, despite best efforts, traditional primary screening will be inadequate to fully characterise some genuine families. For example, it may be impossible to prove one or more genuine pancreatic cancers, meaning that a family would be classified as 'FPC query' and would therefore, not be considered for the screening study. Also some obviously high risk individuals may be unwilling to enrol in screening or may wish to avoid some aspects of screening such as the ERCP element. In support of this, the one individual on the EUROPAC registry who may have developed pancreatic cancer within a screening window despite investigations did not have pancreatic juice analysis.

Thorough primary screening and risk stratification is difficult in its own right, but is only the first step. As stated previously, despite considerable efforts over many years

at several screening centres, prospective cancers have been missed but not detected (at a curable stage) by the early screening studies.

It is these difficulties with both primary and secondary screening that made the risk stratification model attractive. Before the risk stratification project was started, the classification committee would make a subjective estimation of risk based on the number of affected individuals within a family, the total number at risk and the proximity of the nearest affected individuals to the key family members. Now that the risk stratification model has been completed, the simplest method of making this estimate of risk is via the online template. As prospective cancers are detected and the risk stratification model is proven with prospective data, the decision to include participants on the screening programme will become less subjective and based on quantitative estimation of risk. At present, it is possible for a member of a FPC kindred to enter the secondary screening study even though their nearest effective relative may be, for example, an uncle and the age of their parents makes it highly unlikely that they are a gene carrier. Screening such individuals exposes them to the risks of screening and increases the costs of detecting what will be a very low proportion of pancreatic cancers.

One simple step to increase the cancer risk amongst the screened cohort in the secondary screening study is to limit entry to the study to families with three or more pancreatic cancers until the methods and protocols have been optimised. This would, however, exclude more than 2/3 of EUROPAC's FPC kindreds from screening and obviously raises the prospect of a prospective cancer occurring in a

family not considered at sufficient risk for the secondary screening study. The entire process raises serious ethical considerations. Those related to primary screening will be discussed in the following section, with those related to secondary screening discussed later in the relevant part of this chapter.

4.1.2 The Ethics Surrounding Primary Screening

The identification of high risk groups is not straightforward. Individuals normally contact EUROPAC as they are conscious that their family history may heighten their own risk of pancreatic cancer. At the end of the primary screening process, the research team often confirms an elevated risk, but until the generation of the computer model, was unable to quantify this on an individual basis. With a genetic test only available to a minority, one was left using estimates of risk derived from the work of Klein *et al*[185] and this imprecision often increased rather than alleviated anxieties.

There are some advantages to undergoing the primary screening process. Primary screening makes no difference to an individual's inherited cancer risk, just their own awareness of it. Access to information may help individuals rationalise their fears and adopt risk minimisation strategies such as smoking cessation. High risk individuals identified by primary screening may enter the secondary screening study which gives a rational focus and potential solution.

If EUROPAC only made people aware of their heightened risk, without offering access to any form of screening, the ethics of the whole process of primary screening would be called into question. Until the secondary screening protocol has been proven as capable of detecting early pancreatic cancers at a curable stage, increasing anxiety without a proven solution means that the justification for primary screening is inadequate.

High risk individuals have, by definition, personal experience of pancreatic cancer. They are often highly motivated to participate in research and it is a challenge to make sure that the decisions they take are objective. If screening for pancreatic cancer is to be developed, it must be done in the group most likely to benefit. This makes it inevitable that the high risk group be identified.

EUROPAC can act as a source of information about pancreatic cancer but is unable to offer either proven screening or prophylactic surgery. Individuals react in different ways to this situation. Some decline to register and refuse any future contact. Optimistically we can say these patients return to their original state of anxiety. Most kindreds register with some entering and others declining to enter the secondary screening study. In either case there is a presumed benefit from discussion of their anxieties. A small group of individuals may or may not register, but find it difficult to cope with the conflicting evidence and advice regarding screening. They often have a high level of anxiety, which may have been exacerbated by their contact with EUROPAC. They may require considerable input from the research team with questionable advantage to either the researchers or the individuals concerned.

4.1.3 The Differences Between FPC and HP

In those that meet the criteria for definition as a FPC kindred (and the cancer cases in the family have been confirmed), lifetime risk approaches 100% in carriers equating to approximately 120 fold that of the general population (see figure 13). A genetic test is only possible in a handful of FPC families where a *BRCA2* mutation has been identified.

This contrasts with HP kindred where the vast majority can have their diagnosis proven by a relatively simple genetic test and due to previous work by this group and others, the attached risks are well characterised for most individuals.

The level of anxiety about cancer risk is family (and individual) dependent and no differentiation can be made between FPC and HP kindreds. There are other differences that are particularly relevant to secondary screening and these will be discussed in section 4.3.

4.2 Risk Stratification

The low number of prospective cancers and the absence of curable pancreatic cancers detected within the early screening studies has prompted a renewed focus on risk stratification. The work done as part of this thesis will be discussed with reference to both FPC and HP kindreds.

4.2.1 The Role of the Mathematical Model

The mathematical model has the potential to be a significant step forward in risk stratification between kindreds and selection of individuals from within these families for screening. However, before it can be used for this, it needs to be proven using prospective cancers, rather than the retrospective dataset used for its construction. Despite this limitation, it is already a useful tool for patient counselling, being a definite step forward from the Klein's familial risk calculations[185].

The creation of a new tool for risk stratification and counselling raises the question about how best to use it. As already described, traditional primary screening and risk stratification has been limited to a few research centres. The model and online interface offer the possibility of broadening access and raise questions as to how this is best done. The options are: to limit access to the interface to the large research centres and effectively continue with the present system; or we could give access to hospital based pancreatologists, all hospital consultants, all medically qualified personnel including GPs, or make the interface accessible to the general population. There are advantages and disadvantages to each scenario but I favour restricting

access to consultant pancreatologists, at least until the model has been proven using prospective data and the protocol for secondary screening for high risk individuals has been validated. These pancreatologists would preferably all be EUROPAC collaborators who would complete the assessment during an outpatient consultation. This should ensure quality control and mean that newly identified high risk individuals have specialised counselling available as their heightened risk is confirmed.

Once the model and screening have been prospectively proven, online access to the risk stratification model could be opened up to primary care physicians as a tool to help them counsel patients. It could also help GPs decide which individuals to refer on for potential recruitment to the screening study. It is likely that a GP would require an initial double appointment supported by further consultations when completing the template and providing counselling to a high risk individual. It may be that they would prefer this to be undertaken by EUROPAC as it will not be simple to accommodate this given the time constraints operating in most UK primary care facilities.

4.2.2 The Role of Serum Glucose

The role of glucose as a potential modality for either diagnosing or stratifying risk of pancreatic cancer remains unproven in both the general population as well as high risk groups. It has been established that hyperglycaemia can be a symptom of serious, often malignant disease[237]. The data and provisional results included in this thesis have not disproven it as an early marker of pancreatic cancer, but it remains unclear whether it is of any more utility than other markers (such as CA19-9), which can only reliably detect pancreatic cancer when it has reached an incurable stage.

The data collection that has been performed as part of this thesis offers the opportunity to analyse whether a raised serum fasting glucose level is associated with either PanIN lesions or early cancer. However, in the absence of either detected cancers or pre-cancerous lesions in the FPC arm of the secondary screening study, the data need time to mature and any analysis will have to wait until at least some have been detected.

The work displayed on glucose in the results section of this thesis is weakened not only by the lack of detected lesions, but is confounded in the HP arm by the incidence of pancreatitis associated endocrine pancreatic failure.

The data from sporadic pancreatic cancer cases presented in this thesis suggest that hyperglycaemia is a result of the presence of a pancreatic ductal adenocarcinoma, but not cholangio- or ampullary carcinoma and that it resolves after surgery in the PDAC group but not in the other groups. This is in keeping with the findings of other

centres[132, 234] and although the reasons for this cannot be proven, the theory of diabetogenic peptides being released from PDAC is attractive, with S100A8 remaining a possibility[131]. In any event, pre- and post-operative glucose levels do not affect survival irrespective of the tumour type resected.

The data on sporadic cases in this thesis are biased by the lack of information on diabetic status, length of diagnosis and medication taken (both pre and post-operatively). Despite these biases (which affect all three groups equally), the degree of resolution of hyperglycaemia after surgery in the PDAC group is so overwhelming and obviously different to the other two tumour types that the findings are almost certainly not an artefact.

In the future serum glucose may be shown to have utility as a tumour marker, with new onset diabetics being a potential high risk group for screening. At present, however, with screening protocols remaining at an early stage and no cancers yet detected and cured in existing higher risk groups, extending screening to lower risk groups must remain something for the future.

4.2.3 Improved Characterisation of the Phenotype of p.A16V

Prior to this thesis analysis of the p.A16V mutation of *PRSS1* had been avoided, probably due to the low number of available data. This challenge was overcome by recruiting as many mutation carriers as possible worldwide. This approach led to new challenges which will be discussed in the following sections.

4.2.3.1 Recruitment

EUROPAC has traditionally recruited individuals with a family history indicating a genetic predisposition to pancreatitis, prior to any genetic testing. This approach did not prove adequate to characterise p.A16V and active recruitment was required from Europe and elsewhere, to identify any individual with a p.A16V mutation. This is an obvious departure from EUROPAC's traditional recruitment policy and could have introduced bias to the data.

Individuals with p.A16V mutations have been recruited to EUROPAC since the early days of the registry, but it has been difficult to define cancer risk and rationally include carriers in cancer screening due to the low number of individuals affected by pancreatitis and the even lower number of cancers in these kindreds. HP clearly has an elevated risk of cancer that far exceeds the risk associated with idiopathic chronic pancreatitis and in this thesis I have argued that HP patients represent a suitable group for research based screening but sporadic pancreatitics do not. The question that must be addressed is whether the pancreatic cancer risk in p.A16V kindreds is closest to that of HP kindreds (justifying screening); affected individuals with

sporadic pancreatitis (who are currently not screened); or a completely new category with a new definition. The p.A16V mutation has mainly been described in idiopathic pancreatitis[188, 191, 216] but in this thesis I have shown that it can also have a phenotype consistent with both HP and compound recessive disease.

4.2.3.2 Comparison of p.A16V with Other Mutation Groups

The low number of p.A16V families, combined with the different referral pattern for this mutation, made comparison of disease severity between p.A16V and the other mutations difficult. Hierarchical analysis has been used in other studies to allow for family structure[92] but low numbers prevented this in p.A16V. Relatively simplistic statistical approaches were adopted for analysis of the p.A16V data and this must be taken into account when considering the results, but the great variability in penetrance for this mutation is self evident.

The most likely cause for the variability in phenotype is that the p.A16V mutation contributes to a multi-gene effect, whereas simple autosomal dominant aetiology is accepted for both p.R122H and p.N29I related pancreatitis. Multigene dependence may explain the trends seen for both later onset of pancreatitis and diabetes in p.A16V but the low numbers make definitive conclusions difficult. There is the possibility that rather than being a purely genetic phenomenon, there is an interaction between genetic and behavioural factors, such as smoking, but this remains unproven with the data being too few to come to a conclusion.

4.2.3.3 Mechanism of Action of p.A16V

As stated previously, p.R122H and p.N29I have both been linked to either increased auto-activation (or reduced deactivation) of cationic trypsinogen[217, 218]. Phenotypically, p.R122H and p.N29I are very similar, although p.R122H results in a slightly earlier age of onset as shown in figure 8 of this thesis and in previous reports using EUROPAC data[92]. The p.A16V mutation lies at the edge of the signal peptide of trypsinogen and has previously been considered to influence secretion[191]. It is tempting to assume that a secretion defect is inadequate to cause pancreatitis without some other genetic or environmental factor being involved, hence the difference in phenotype. The mis-folded protein response associated with secretion failure is considered to explain the link between the p.R116C mutation of *PRSS1* and pancreatitis but so far this mutation has only been linked to autosomal dominant disease[325]. In addition, the latest available data indicate that the p.A16V mutant is actually secreted normally[325]. Other work has established that p.A16V increases the rate of chymotrypsinogen C (caldecrin) activation of trypsinogen by approximately four-fold. This results in accelerated trypsinogen activation *in vitro*, possibly explaining the link with pancreatitis[326]. How this mechanism could result in the variable penetrance seen in p.A16V remains unclear.

Some families do appear to have a phenotype consistent with HP. This suggests relatively common polymorphisms in modifier genes within the populations that contribute to these families, although it remains possible that this is explained by a shared environment. One family has highly aggressive disease restricted to a single

generation, suggesting a 'jackpot' combination of the p.A16V mutation from one side of the family and polymorphisms in modifier genes from the other. Relatively few families were identified with single cases of pancreatitis but it is likely that a higher proportion of such families go unreported and most will never undergo genetic testing. Penetrance in such families may require specific environmental exposures and the low number of individuals recruited with a phenotype consistent with sporadic disease may be the result of EUROPAC's traditional recruitment criteria.

4.2.3.4 Cancer Risk and p.A16V

Prior to this thesis, almost nothing was known about the pancreatic cancer risk associated with the p.A16V mutation. The presence of three cancers in ten p.A16V kindreds are fewer than would be expected if the risk in p.A16V kindreds were the same as in p.R122H and p.N29I families and it is noteworthy that there were no cancers in the families with a phenotype consistent with sporadic disease. The data are, however, too few to issue specific guidance on whether individuals from p.A16V kindreds should be screened. In terms of current clinical management, it would seem sensible to manage p.A16V kindreds by phenotype, offering access to the screening study to those with a phenotype consistent with HP, but to not offer it to those whose phenotype is consistent with sporadic disease. This can obviously be reviewed as more conclusive data become available.

4.3 Secondary Screening

Setting up the secondary screening studies raised a large number of ethical and management issues that will be discussed in the following sections.

4.3.1 Ethics

Obtaining the national ethical approvals for the studies of secondary screening had been discussed within EUROPAC for several years and as such, was a significant step forward. Going through the process required to obtain national ethical approval meant that the foreseeable ethical issues were considered and solved in advance but any large trial will inevitably lead to ethical dilemmas that arise on a prospective basis. Both anticipated and prospective ethical dilemmas will be discussed in this section.

4.3.2 Prospects for Success

It is of interest that there are now five studies of screening for early pancreatic cancer in high risk groups. If the data were pooled, there would be several hundred years of patient follow up, but the first curable cancer has yet to be detected. This raises the possibility that the level of risk within the study populations does not justify either the monetary cost or health risks associated with secondary screening.

The most likely reason for this is inadequate primary screening. When any research group is launching a study, both potential participants and the research teams would have been keen to start the screening process. It is possible that some individuals were entered into the studies where the diagnoses of pancreatic cancers in families were thought likely but could not be completely proven, meaning that some individuals from kindreds with a normal risk of pancreatic cancer may be participating in the screening studies, albeit quite happily. I have already described that EUROPAC withdrew one individual from the FPC screening study for exactly this reason and that a second individual from a HP kindred was screened before the age of 40 years. This increases the number of investigations required to detect a cancer, increases costs per cancer detected and has implications in terms of complications and side effects caused by unnecessary screening.

A second important method by which to judge the screening protocol is whether it fails to detect a cancer that is emerging within one of the patients that is being screened. It is important to maintain the screening intervals and minimise patients lost to follow up to try to prevent this eventuality. As has been described, no

prospective cancers have been missed in the EUROPAC cohort undergoing the full screening process, although a possible pancreatic cancer did develop in an individual at the periphery of the screening programme. Both the University of Washington and John Hopkins studies have described interval cancers and there is no place for complacency.

A screening program should be judged on the basis of how many investigations and years of screening will have to be undertaken to detect each cancer, the costs incurred, both financial and in terms of adverse events, and whether the resultant surgical treatment can prove curative. Even using 2006 figures[300] EUROPAC has already spent more than $110 000 on imaging alone and has yet to detect a cancer. Determining the success of treatment will require at least five years follow up after surgery and therefore falls beyond the scope of this MD project, particularly given the rate at which abnormalities are being detected.

4.3.3 The EUROPAC Secondary Screening Protocol

There is minimal evidence to justify screening for pancreatic cancer by blood testing in isolation. However, minimally invasive methods of screening are attractive, and in time, the data gathered as part of the secondary screening studies may make it possible to show a correlation between changes in blood results and abnormalities detectable on imaging. However, the detection of several prospective cancers will be needed before the significance of blood tests performed as part of a screening program can be quantified.

I would argue that EUS is the best way of imaging normal pancreatic tissue. It is minimally invasive and generally well tolerated. The results section of this thesis show compliance in the FPC group to be 93% compared to 79% in the HP group. It is the primary imaging modality in the FPC group (where the pancreatic tissue is normal) and delivers excellent, if user dependent, images. The main drawback with EUS is its inability to differentiate cancer from acute or chronic lesions in individuals with severe chronic pancreatitis or post-surgical change. This severely limits its use in the HP sub-group and EUROPAC does not use EUS as the primary imaging modality in these individuals, reserving it for corroborative imaging of lesions shown by other modalities and for guiding FNA if recommended by the pancreatic MDT.

The use of CT scanning in preference to MRI deserves an explanation. CT has been proven to be able to detect early pancreatic cancers and this has yet to be done in the case of magnetic resonance imaging. MRI has definite attractions, principally that it does not involve the delivery of a dose of ionising radiation to the study

participants, a proportion of which are known to have DNA repair mutations. However, the aim of this study is to detect early pancreatic cancers. Even if there are risks attached and the screening interval needs to be carefully considered, it is important to use the modality which is most likely to detect an emerging cancer, particularly in view of the prognosis if diagnosis is delayed. When screening *BRCA2* mutation carriers, where the mutation prevents the repair of genetic abnormalities, there is a stronger case for using MRI as the primary imaging modality. One must at least attempt to avoid or minimise the generation of new potentially non-repairable DNA abnormalities by the ionising radiation involved with CT. As the technologies continue to improve and research continues, MRI may be shown to be equally as effective as CT in detecting early pancreatic cancers. The lack of ionising radiation would then make it the non-invasive imaging modality of choice.

The use of ERCP has both advantages and disadvantages. Potentially, molecular analysis offers the best chance of detecting lesions that are progressing towards PDAC whilst they are still at a pre-malignant stage. It informs the screening interval in the EUROPAC screening protocol indicating which at risk individuals merit particular attention. If molecular analysis is proven in a prospective trial to indicate emerging pancreatic cancer it would become ethically justifiable to remove a pancreas on the basis of molecular results alone. It is important, however, to differentiate between the resection of an indeterminate lesion in the presence of molecular abnormalities and a second scenario of operating where genetic abnormalities are present in pancreatic juice, but imaging indicates a normal pancreas. The role of molecular analysis could yet take on particular significance in

the HP group where the role of imaging is limited by the chronic inflammation and the risk of post-procedure pancreatitis appears low. However, the two operations performed so far were both in HP patients with multiple pancreatic juice abnormalities. Histology results indicated that one had PanIN 2 lesions and the other had a normal pancreas, showing that it is possible to detect multiple genetic abnormalities, including a *Tp53* mutation, in the pancreatic juice harvested from a histologically normal pancreas. Inevitably it is impossible to know in such patients whether the molecular markers were genuinely indicative of a nascent cancer and if this were the case, how long it would have taken for this tumour to develop. The only way to be sure of this would be to follow patients with mutations detected in juice without any surgical intervention. There are obvious ethical difficulties associated with such an approach.

Any endoscopic procedure brings a risk of perforation but the real drawback of ERCP is the potential for post-procedure acute pancreatitis[279]. The numbers performed remain too small to make meaningful statistical analysis possible, but whilst the risk in the HP sub-group, (where inflammation takes place on a chronic basis) appears small, the risk in FPC patients is very real. There were no cases of pancreatitis amongst the 16 ERCPs performed to August 2008 in the HP group. In contrast post-procedure pancreatitis affected 6/16 individuals in the FPC group, with just 11 of those procedures successfully obtaining a sample of pancreatic juice.

4.3.3.1 How the Screening Protocol has Evolved

Prior to the national research ethics application, screening in high risk groups had already been initiated using existing local ethical approvals. This took place on an evolving basis at several centres. The national application brought several issues into focus. Primary screening was improved to increase the probability that individuals were at genuine risk before investigations were initiated and the secondary screening protocol was first adjusted and then followed. This was both to minimise the chance of missing an evolving cancer and to avoid unnecessary screening. The protocol will continue to need adjustment as prospective cancers develop.

The collection of fasting glucose was added in light of the increasing interest in glucose prompted by the results of other groups, particularly those based at the Mayo Clinic[236, 327, 328] . Screening intervals became more formalised with the definite adoption of EUS, followed by CT, as the imaging modalities of choice in the FPC group. Initially, any genetic abnormality in the pancreatic juice prompted further ERCP for a repeat sample. As the number of results increased, single Kras mutations were accepted within the standard surveillance pathway, although an abnormality in *CDKN2A* or *Tp53* still triggered the individual being identified for entry to the close surveillance pathway.

After the national ethics application was approved, the only change to the protocol shown in figure 4 during my period of research was that rectal diclofenac was

adopted as prophylaxis against post-ERCP pancreatitis in the FPC group. There are a range of factors that have been implicated as being associated with post-ERCP pancreatitis[245, 278]. These can be grouped as patient factors and technical factors related to the procedure. The evidence linking diclofenac to prophylaxis of pancreatitis is mixed[280, 281] and the screening cohort offers an opportunity to investigate this further. Following the introduction of diclofenac prophylaxis, there were no further cases of pancreatitis during my time in post.

An application for an amendment has since been granted to collect duodenal juice after administration of secretin to compare molecular results with and without cannulation of the pancreatic duct. This should eradicate the risk of pancreatitis but is beyond the scope of this MD project and is very likely to form part of my successor's thesis.

4.3.4 Problematic Screening Scenarios

As mentioned above, the screening study led to both the identification of obvious but also some unanticipated ethical and management problems and these will be examined in turn.

4.3.4.1 Differences Between FPC and HP Kindreds

Individuals from FPC kindreds generally have normal pancreatic parenchyma. The results section of this thesis shows that of the 70 EUROPAC individuals from FPC kindreds screened to date, just two suspicious abnormalities have been detected. Imaging normal tissue maximises the sensitivity and specificity of imaging and molecular analysis should allow more accurate stratification of risk, but there are two major disadvantages.

Firstly, individuals from FPC kindreds have an increased risk of post-ERCP pancreatitis. There were six cases of pancreatitis out of the 16 ERCPs performed in the high risk individuals from FPC kindreds and pancreatic juice was only obtained from 11 of the procedures. Whilst diclofenac may reduce the risk and sampling duodenal juice after administration of secretin may remove it, this is a potentially serious problem that requires careful thought. The causes for this apparent trend towards more post-ERCP pancreatitis in this group are unclear, although it could be due to cannulation of a normal pancreatic duct.

The second issue is that the generally normal pancreatic parenchyma in the FPC group means that any false positive result from multimodality screening would

potentially prompt the resection of normal pancreatic tissue, with the potential for post-surgical endocrine and exocrine pancreatic failure, depending on the procedure performed.

In contrast screening of high risk individuals with HP brings its own specific problems. The available imaging is far less specific due to the presence of chronic pancreatitis, with the low specificity of EUS in the HP group, prompting the adoption of CT as the primary imaging modality, despite the associated dose of ionising radiation. The stratification offered by molecular analysis is also less marked as at least some of the genetic changes used to stratify the risk of an existing or evolving cancer can be found in the pancreatic juice of chronic pancreatitics[220]. ERCP appears safe in this group. Of the 16 ERCPs performed, there was one patient that experienced post-procedure pain, but there were no cases of pancreatitis. It is possible that, as more data are collected, the molecular analysis of pancreatic juice could take on a greater relative significance in this group compared to FPC due to the problems with imaging, although this statement cannot be supported from the limited evidence currently available. Case A has shown that molecular changes as advanced as a *Tp53* mutation can be detected in pancreatic juice from what is subsequently shown to be a histologically normal pancreas. Case B has shown that it is not easy to differentiate between inflammatory and malignant cystic lesions. This makes it likely that the histology from a higher proportion of operations performed in the HP group will subsequently show benign disease, although in Case B's case, PanIN lesions were present.

The disadvantages of screening HP individuals are offset by the high incidence of exocrine and/or exocrine pancreatic failure in this group. It is common for pancreatic failure to result from pancreatic resectional surgery[329]. The Howes paper[92] showed the cumulative risk (95% CI) at 50 years of age for exocrine failure to be 37.2% (28.5%, 45.8%) and 47.6% (37.1%, 58.1%) for endocrine failure. The two resections performed during the period of this MD project have both been on HP patients with pre-existing exocrine pancreatic failure.

Pancreatic resections carry a significant risk of both mortality and morbidity, but at least when resections are performed, the excised tissue would generally be expected to be of chronically diseased, rather than normal tissue - although as case A shows, this will not always be the case.

4.3.4.2 Screening Commencement and Cessation

Figures 13 and 21 show that although the risk of pancreatic cancer increases with age, it is very low below the age of 40 years even in high risk patients, but then rises steadily thereafter. EUROPAC's decision to start screening from the age of 40 years results from this analysis. There are arguments for and against taking 40 years as the starting age for screening. If one starts screening earlier than the age of 40 years, one exposes individuals to unnecessary ionising radiation. Delay raises the possibility of a missed cancer.

Any start point is potentially controversial as it is by definition arbitrary. Cancer risk will gradually increase. It will neither be zero at the age of 39 years nor maximal at the age of 40.

It is important to try and stratify risk within high risk kindreds on an individual basis. Individual risk is obviously the most important factor in determining the point at which screening should commence. It can often only be estimated, even after thorough primary screening, although the risk stratification model should aid in this.

There must also be sufficient flexibility to allow for special circumstances. Some FPC kindreds contain proven cancers that have occurred before the age of 40, with the youngest diagnosis known to EUROPAC being at the age of 29 years. Commencing screening from 40 in this kindred would cause both a great deal of anxiety to the individuals concerned and risk missing a cancer. Similarly, the existence of anticipation amongst EUROPAC FPC families has also been identified[101] and must be taken into account in the multi-disciplinary discussion that takes place at the end of the primary screening process.

In HP kindreds 40 years is also the starting age for entry to the screening study. EUROPAC data, which were initially presented by Howes *et al*[92] and also included in a review of HP by Vitone *et al*[244], show that the cumulative risk of pancreatic cancer in HP kindreds rises from 0.5 to 3.4% between the ages of 40 and 50 years. Cancer risk rises exponentially thereafter reaching 33.3% at the age of eighty years. The cancer risk at each age must be balanced against the risks involved with an experimental screening protocol involving the use of ionising radiation.

The EUROPAC secondary screening programme is at an early stage, but has not missed a cancer so far. On the basis of this (albeit limited) experience, the screening programme should continue to start from the age of 40, unless the family history indicates a particularly high risk. Flexibility will be required as prospective cancers occur both inside and outside the screening population.

The age at which screening should commence is therefore supported by the available statistical data, for both FPC and HP kindreds. The age at which screening should cease is, however, more difficult. The published data[92, 244] and figure 21 of this thesis show that cancer risk increases with age. The year on year risk of an individual from an FPC kindred developing cancer begins to decline after the age of 67, with those unaffected by pancreatic cancer having an increasing chance of not being mutation carriers for each subsequent year that they remain unaffected. Sporadic cases of pancreatic cancer will also occur in high risk families with an incidence that increases with age. In the HP kindreds no link has been shown between cancer risk and the number of years of pancreatitis, but there is no specific age when the risk of cancer starts to reduce.

Choosing the time at which to stop screening is ethically complex. All screening programmes have a date at which screening stops. This may be due to risk-benefit or financial factors. It is clinically and ethically difficult to screen somebody for early pancreatic cancer for many years and to stop this, for example when an individual reaches the age of 70. At present the decision on when to stop screening in both FPC and HP kindreds is being made by an individual's consultant clinician and is

largely determined by the individual's ability to withstand major pancreatic surgery were a tumour to be discovered. However, this is one area that needs to be subjected to ongoing re-assessment as the screening studies continue. On the basis of experience with secondary screening to date, there is no indication to change the current policy and decisions should continue to be made by responsible clinicians in conjunction with the screening participant.

4.3.4.3 Screening Individuals with Post-surgical Changes

A further dilemma that should be considered is whether to attempt to screen individuals, predominantly from HP kindreds that have had a previous partial pancreatectomy. In time, this scenario may also become relevant to individuals from the FPC group that have already had a Pylorus Preserving Kausch-Whipple procedure (PPKW) for a pancreatic tumour. At present there are insufficient data to make an evidence based decision, but if one accepts the PanIN theory and PanINs have been shown to develop throughout the pancreas, it is logical that tumours could arise within the pancreatic remnant. In terms of screening this raises several problems that will be discussed in the following paragraphs.

Firstly it would be difficult to detect an emerging lesion in the presence of post-surgical change. If a new lesion were to emerge, imaging would probably detect it later and struggle to differentiate between possible aetiologies. In cases where there are problems with imaging, the molecular analysis could take on a greater significance, but as figure 23 shows, the surgical reconstruction performed as part of a pylorus preserving Kausch-Whipple (PPKW) procedure means that the duct is not readily accessible with an endoscope. Whether juice could be collected from these participants after secretin and whether its analysis would mean anything remains to be proven.

Figure 23: Reconstruction of Gastro-intestinal Tract after Whipples Procedure

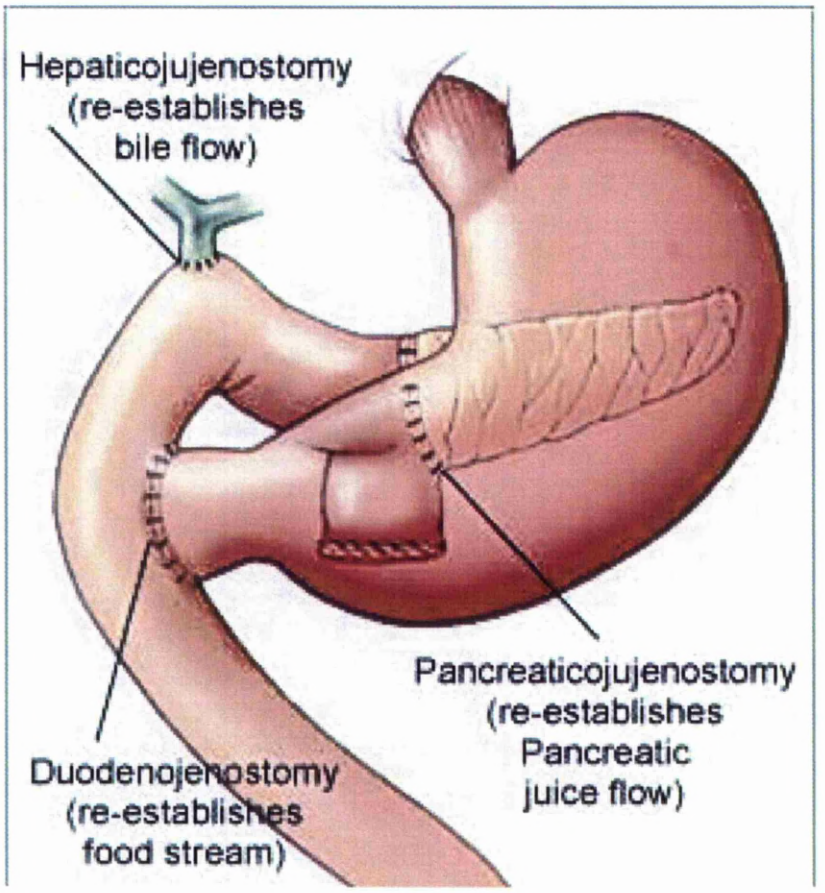


Figure illustrating the reconstruction of the gastrointestinal tract performed following the resection of the pancreatic head performed during a pylorus preserving Kausch-Whipple procedure (PPKW). Copyrighted and used with permission of Mayo Foundation for Medical Education and Research, all rights reserved.

The secondary screening programme has, as yet, gathered too few data to give a firm recommendation on screening pancreatic remnants for early cancers but I feel that collaborating clinicians should continue to make screening decisions on an individual basis and data collection should continue in these challenging participants.

4.3.4.4 HP Screening Dilemmas

This section contains a discussion on screening in three different but related groups of individuals either with pancreatitis or from HP kindreds. They will be considered in turn, but my attitude to screening all three groups is similar.

Should Those with Chronic Pancreatitis be Screened?

Those with chronic pancreatitis have long been known to have an elevated risk of pancreatic cancer[134, 330, 331]. As outlined in the introduction, there are also mutations of the *SPINK1* and *CFTR* genes that are associated with the development of pancreatitis, rather than being causative mutations in the true sense. The cancer risk in chronic pancreatitis from a non-HP aetiology[62] has been estimated at 15%, obviously far lower than in true HP (see figure 21) or FPC (see figure 13). Screening is proving challenging enough in the groups at highest risk and my opinion is that those at lower risk should not be put through the rigours of a screening study until the protocol has been optimised. Once the specificity of multi-modality testing has been optimised, screening may become justifiable if the potential number of lives saved outweighs the morbidity and mortality of intervention on the inevitable false positives.

Should Unaffected Carriers of HP Mutations be Screened?

The calculation of lifetime risk of pancreatic cancer in individuals affected by HP has been calculated at 35-53%[91-93]. Cancer in HP mutation carriers unaffected by pancreatitis is very rare but the diagnosis of two cancers in separate individuals in exactly this position described earlier in this thesis has raised the question as to whether such individuals should be screened.

The exact cause of pancreatic cancer in HP individuals is unproven. Chronic inflammatory change is an attractive theory, but as stated above there is no proven link between cancer risk and number of years of pancreatitis in the EUROPAC population. The diagnosis of cancer in the two asymptomatic carriers also raises the possibility that the aetiology of cancer risk is not exclusively related to pancreatitis.

At present the decision whether to offer screening is being taken by the responsible clinician, who makes an assessment of risk and benefit and the individual's wishes. I am only aware of these two cases of pancreatic cancer in unaffected HP mutation carriers worldwide. The adverse events section of this thesis shows that the screening process is not risk free and my opinion on screening unaffected mutation carriers is similar to that relating to those with non-hereditary chronic pancreatitis. I believe the screening protocols should be optimised in populations with better defined risk before potentially being extended to groups where the risk is lower or less well defined.

Should Affected Individuals with very rare PRSS1 Mutations be Screened?

There are other kindreds with at least one and often several cases of pancreatitis with a proven *PRSS1* mutation which is presumed to be the cause of the disease within the kindred. Although the cancer risk in p.R122H and p.N29I kindreds can be proven, quantifying a cancer risk (or even proving that the pancreatitis is genetic) in kindreds with very rare *PRSS1* mutations is statistically impossible. In kindreds with a p.N29T mutation and a phenotype consistent with HP, it is sensible to manage them in the same was as HP kindreds with a p.N29I mutations. The cancer risk in pancreatitics risk with other very rare *PRSS1* mutations such as p.R116C or p.E79K cannot be quantified. My own opinion is that screening decisions in these kindreds should be similar to those in the p.A16V kindreds, where decisions are made on the basis of phenotype. If the phenotype is consistent with HP or IPCA, then access to screening should be offered. Where the phenotype is consistent with idiopathic disease, then those individuals should be treated in a similar fashion to those with idiopathic or non-HP chronic pancreatitis, with access to the screening study considered only after the protocol has been optimised.

4.3.4.5 What Should be the Indications for Surgery?

Whenever a potentially significant abnormality is detected in a study participant, the case is discussed at the supra-regional pancreatic MDT. Pre-operative decision making is subjected to local peer review in that venue. As long as there is a general consensus from MDT members that surgery is indicated, participants are offered resection. Those from the screening study are treated exactly the same as all other individuals discussed at the MDT, with the main indication for surgery being the presence of a potentially resectable mass on imaging in the absence of metastatic disease. It must be borne in mind that an unknown percentage of the detected lesions in the HP group will be inflammatory, as was the case in the second of the two resections performed to date.

Can Prophylactic Surgery be Justified?

Given the current limitations of screening, one definite way of removing the risk of pancreatic cancer in high risk individuals is prophylactic total pancreatectomy. Those undergoing such surgery would not only face the ≈5% mortality risk of major pancreatic surgery[332] but would by definition require both exocrine pancreatic supplements and insulin for life[333, 334] . The resultant diabetes would often be difficult to control[335]. Prophylactic surgery is a radical step but, given the personal experiences of pancreatic cancer in EUROPAC kindreds, is not an unusual request from individuals contacting the study office.

When discussing prophylactic total pancreatectomy, it is important to differentiate between the FPC group, with their normal pancreatic parenchyma and the HP group with what is normally chronically diseased tissue, with the individual often diagnosed with either exocrine and/or endocrine pancreatic failure. I am unaware of any individual from a FPC kindred that has ever had prophylactic pancreatectomy, but a total pancreatectomy in a HP individual that has already been diagnosed with exocrine and pancreatic failure is less controversial. If there is a proven HP mutation, lifetime pancreatic cancer risk exceeds 40% and individuals may see the 5% mortality risk of surgery as acceptable in an attempt to remove this. In addition it may bring the added benefit of relief from the symptoms of chronic pancreatitis.

Can Surgery be Justified on the Basis of the Results of Molecular Analysis?

The ideal time for surgical intervention would be when development of malignancy becomes inevitable within a pre-malignant lesion but before any emerging tumour either becomes locally invasive or undergoes metastasis. At present this window cannot be accurately defined. In time, the molecular analysis of pancreatic juice may permit identification of a 'pre-malignant window', but the initial results from the screening study have raised more questions than they have answered, with one of the resected pancreata being histologically normal, despite the presence of multiple genetic abnormalities including a *Tp53* mutation in the pancreatic juice. Data collection should continue, but it is unclear how long it will take to show whether the molecular analysis has any clinical role other than the phasing of screening investigations, or indeed, whether this is even justified.

Which Operation Should be Offered?

If participants in the screening study come to surgery, the options are either a Whipple procedure or a total pancreatectomy. In Whipple's procedure, the tail of the pancreas is retained (see figure 23). The morbidity of post-operative pancreatic failure would be reduced but this must be offset against the risk of a further lesion in the pancreatic remnant, which as stated above, would be both difficult to detect and may not be easy to remove. Although it is more common for pancreatic cancers to arise in the head rather than the body or tail[336], it has already been shown that PanIN lesions occur throughout the entire pancreas[337], as was true with Case B described in this thesis. The principal aim of screening and the associated surgery is related to cancer detection and cure. Despite the increased morbidity of post-surgical pancreatic failure induced by a total pancreatectomy rather than Whipple's procedure, resection of the entire pancreas (albeit duodenum and spleen preserving where appropriate), appears the best choice for the majority of patients that come to surgery with early lesions as a result of screening.

4.3.5 Operations so far

To date two study participants from EUROPAC secondary screening study have undergone pancreatic resection as a result of investigation results. The first resection (Case A), described in section 3.3.3.2 is interesting for a number of reasons.

Baseline imaging showed normal pancreatic parenchyma on CT, later confirmed on EUS, but molecular analysis showed all three genetic abnormalities associated with pancreatic cancer to be either present or outside of the normal range. Repeating the juice analysis showed ongoing abnormalities in *Kras* and *CDKN2A* but the second *Tp53* result was Wild Type (normal). Following resection, her pancreatic histology was found to be normal, with no evidence of either PanIN lesions or malignancy.

This obviously raises a few questions. The genetic testing is performed on free DNA that is shed into pancreatic juice following necrosis or abortive apoptosis. It has already been theorised that the presence of *Kras* mutations would be predicted to cause senescence in any cell harbouring the mutation[338], but it is interesting that two of the three *Kras* mutations were not detected and a new mutation was observed in the second test. The main mutation in both cases was p.G12R suggesting a possible clonal population but with a mosaic of the of the K-Ras mutant cells around it, perhaps dying due to lack of protective tumour suppressive mutations. In addition, the p53 test showed a mutation (p.D245G) which was no longer present in a second sample of pancreatic juice. It could be that the cells that had shed the mutated *Kras* and *Tp53* sequences had undergone apoptosis and were no longer present at the

time the second sample of juice was obtained. The data are too few to come to a definite conclusion.

Histological results following Case A's resection showed the presence of normal pancreatic tissue in all the examined blocks, despite a thorough search for PanIN lesions. This undermines the attractive theory that Kras mutations, *CDKN2A* hypermethylation and *Tp53* mutations could be used as markers of increasingly abnormal changes along the metaplasia-dysplasia pathway as outlined in figure 1. Case B, described in section 3.3.3.3, also had both a *Kras* mutation and *CDKN2A* hypermethylation in her pancreatic juice. Her subsequent histology sectioned the entire pancreas. There were numerous foci of PanIN 1a and 1b with possible foci of PanIN2, but no neoplasia. These histological findings are in keeping with the theories summarised in figure 1.

4.3.6 Cost Analysis and Cost Effectiveness

Screening for early pancreatic cancer is not cheap. In an environment where demand for healthcare outstrips the supply, then pancreatic screening requires the diversion of resources from other treatment priorities and it is important that any expenditure delivers an overall health benefit.

The cost of screening for pancreatic cancer in Peutz-Jeghers kindreds has previously been calculated to be $350 000 per life saved[300], although it may be possible to reduce this cost significantly by use of molecular analysis of pancreatic juice. To date EUROPAC has spent at least $113 000 on imaging and pancreatic juice collection, without detecting a cancer. It is likely that the number of investigations required per cancer detected will be reduced as the protocol is optimised, but the true cost of screening for pancreatic cancer will only become apparent after several cases have been identified.

If we consider that there are at least five centres screening high risk individuals for pancreatic cancer and one has yet to be discovered at a curable stage, this supports the FaPaCa conclusion[297] that screening should not be extended beyond the study setting.

Whether screening for early pancreatic cancer ever becomes an NHS service will depend on total cost per life saved once the protocol has been optimised. A national programme would require National Institute of Clinical excellence (NICE) approval and would have to compare with other screening programmes. The UK breast

cancer screening programme has previously been calculated to cost in the region of £25 000 per life saved[339].

4.3.7 Adverse Events

There were two areas where there were significant adverse events related to screening process. These related to the death of one individual from carcinomatosis with an unknown primary and to a number of cases of acute pancreatitis triggered by ERCPs performed for the collection of pancreatic juice.

The death from carcinomatosis was in an individual registered by the Liverpool office prior to my research period. They were referred to a screening clinician nearer to the individual's home address in November 2004 for screening investigations. Her screening results were first seen by the Liverpool team in the late Spring of 2008 when all screening results that had not been copied to the Liverpool EUROPAC office at the time they were performed were being checked and collated (with local site visits where necessary) as part of work performed for this thesis.

The individual had both a baseline EUS and CT in July and August 2005 respectively. The EUS was normal. The CT showed mild heterogeneity of the lateral aspect of the pancreatic head, which was commented on and an MR was suggested. An MRCP performed in January 2006 was reported normal. The individual next had a CA19-9 result of 1726 kU/L in November 2006. A CT was performed that month that showed no interval change from the baseline scan in 2005 and an EUS was repeated in January 2007, which was again normal. The patient was reviewed in the clinic in June 2007. Their major symptom at that point was a change in bowel habit to constipation. A colonoscopy was requested along with repeat tumour markers. The CA19-9 result came back at 6704 kU/L. The individual was subsequently

diagnosed with carcinomatosis with an unknown primary. A biopsy showed adenocarcinoma. She died in 2008 and despite the normal imaging performed in the preceding years, it remains possible that the primary malignancy was a pancreatic adenocarcinoma.

This case was managed by a satellite centre prior to the introduction of the screening protocol summarised in figure 4. If this was indeed a case of pancreatic cancer, my interpretation is that it developed despite 'close surveillance'. It is difficult to make any definite decisions from a single case of possible pancreatic cancer, but along with pancreatic cancers that have developed within the US screening programmes, it raises the possibility that changes on imaging can occur late in the process of cancer development.

The second area that must be regarded as an adverse event is the rate of post-ERCP pancreatitis following pancreatic juice collection. Nationally, the expected rate of acute pancreatitis after ERCP is about 5%[277] and the vast majority of attacks were expected to be mild. Although numbers were too small for definitive conclusions, at one point the rate of post-ERCP pancreatitis in the FPC group reached 50%, with one case of non-infected pancreatic necrosis and subsequent exocrine pancreatic failure. To exacerbate this, it was not always easy to collect the pancreatic juice. Even at the end of my research period, of the 16 ERCPs performed, just 11 led to successful juice collection. The post-ERCP acute pancreatitis in the FPC secondary screening population prompted the research team to contact the responsible research ethics committee to ask for permission to use diclofenac per rectum as

prophylaxis against pancreatitis. This was adopted on an interim basis after the 12th ERCP and there were no further episodes of pancreatitis in the subsequent four cases to the time of guillotine.

The majority of screening patients are intelligent and articulate and sought out EUROPAC to gain access to secondary screening. The risks and benefits of each test were outlined in the patient information sheets and whilst I did not collect data for this, my own experience was that most individuals wanted to discuss these risks in detail before consenting to undergo them. They were particularly interested in discussing the balance of risk and benefit with regard to ERCP, where the patient information sheet outlined the potential risk of pancreatitis, but the immediate and direct benefit to the individual was less tangible than with imaging investigations.

My opinion is that data collection should continue in consenting members of both the FPC and HP kindreds, but if the molecular analysis of post-secretin duodenal juice can be validated, the post-ERCP pancreatitis problem will have been permanently resolved.

4.3.8 The Balance of Risk and Benefit

Most members of FPC and HP kindreds are enthusiastic about accessing the screening study but the benefits remain unproven. To date, no pancreatic cancer has ever been detected in an organised screening study at a curable stage. It remains to be shown whether screening will lead to earlier treatment and whether this can prove curative.

The risks of the screening programme need to be considered carefully. These are physical and psychological. The psychological risks involve the heightening of anxiety and confirmation of a diagnosis with a genetic syndrome may lead to a reduction in self esteem or even depression. The diagnosis could lead to discrimination from insurers and employers, but there are also physical risks that are potentially even more serious. Blood testing risks are minimal but the imaging needs more consideration. EUS is the single test that is most likely to discover an early pancreatic cancer but it carries the risk of perforation of a hollow viscus. The risk of perforation from an endoscopic procedure is low[279] but if it did occur it could be life threatening and surgical intervention would probably be necessary. The use of repeated CT scans exposes the participant to the risk of ionising radiation, which has been estimated by the US Federal Drug Agency as 10 Milli-Sieverts (mSv) per abdominal CT scan[268]. To put this into perspective, the UK legal dose limit for adult whole-body exposure is currently 20 mSv/year, but in 1997 the average annual dose in the UK nuclear industry was around 0.6 mSv[340, 341].

The collection of pancreatic juice at ERCP contains an element of risk as discussed previously. The risk of pancreatitis after ERCP is thought to be in the region of 5%, although it was much higher than this in the FPC sub-group until diclofenac was introduced. The most severe case of pancreatitis induced through screening developed sterile necrosis and exocrine pancreatic failure. At worst, ERCP could lead to infected pancreatic necrosis and even death.

Molecular analysis may permit a reduction in the screening interval and total radiation exposure, but it remains possible that the reduction in mortality from pancreatic cancer screening would need to be offset against one or more deaths from acute pancreatitis. Both the University of Washington and Johns Hopkins groups have previously used ERCP for imaging in the search for PanINs. Johns Hopkins have discontinued ERCP for imaging as it was felt that the risks outweighed the gains[295]. The above risks and benefits are not simple to analyse and quantify but they will need to be considered on an ongoing basis as the project develops. As stated previously, the EUROPAC secondary screening study, has yet to detect a cancer. The use of ERCP has avoided some radiation load from annual investigations, but it could certainly be argued that the six cases of pancreatitis induced so far exceeds the benefit derived.

4.4 A Reassessment of the Aims and Objectives

The aims and objectives are laid out in section 1.8 but in summary the first aim was improving risk stratification on an individual basis in both the FPC and HP groups and required: further characterisation of the phenotype associated with *PRSS1* mutations; investigation of whether serum fasting glucose levels can differentiate between individuals from FPC kindreds with differing risk profiles; the development of a computer model capable of stratifying risk in high risk individuals from FPC kindreds.

The second aim was piloting a trial of secondary screening in high risk individuals and required the achievement of set objectives namely: obtaining the ethical approvals; developing a multi-centre collaborative screening network; continuing with effective primary screening to identify high risk individuals; and the testing and development of the EUROPAC secondary screening protocol.

The first aim (and associated objectives) was achieved. If one is aiming to screen individuals for cancer, risk stratification on a familial level alone is unsatisfactory and the ideal must be to perform this on an individual basis. As previously stated the cancer risk in the more common *PRSS1* mutations is well established but clinical characterisation of the p.A16V mutation had never previously been attempted. The investigation of the relationship between serum fasting glucose and differing risk profiles has not shown significant results to date and achieved less than I had hoped for at commencement of the project. The main limiting step was the lack of early lesions detected in the screening study, but data collection is ongoing and as

prospective cancers develop, retrospective trends in serum glucose results may identify a new modality for early diagnosis of pancreatic cancer. The final aspect of improving risk stratification on an individual basis in FPC was the development of the mathematical model and its online template. This had been done before in Breast-Ovarian cancer but had never been attempted or achieved in FPC. The format adopted means that the model can be adjusted and improved as more data become available and prospective cancers develop. It has considerable potential in primary screening, risk counselling and identification of individuals for secondary screening. I believe that this will become a part of routine clinical practice in this specialised field.

The second aim (and associated objectives) was again either fully or partially achieved. The trial was piloted, the ethical approvals were granted and both existing and new collaborators were utilised to deliver national coverage to permit individuals to enter the screening study, with individuals screened as close to their home address as possible. Primary screening continued with 114 new families recruited, with both new and existing families becoming better characterised. The quality of the data collated and stored was improved, cancer deaths were proven more effectively and only genuine families that met all the inclusion criteria were permitted access to the secondary screening study. Within the screening studies: collection of glucose data was added; imaging intervals were strictly defined and delivered; and as greater numbers of juice analysis results became available, single Kras mutations were accepted within the standard surveillance pathway. The testing and development of

the EUROPAC secondary screening protocol was somewhat limited by the failure to detect the first ever pancreatic cancer as part of an organised screening programme, but accepting these limitations, the protocol has developed into a deliverable and testable structure that is delivered through a number of regional screening centres. Prospective cancers will occur in the screening population. It remains to be proven whether these will be detected at a curable stage and whether the financial cost of each cancer detected (and/or life saved) will lead to an expansion of screening for early pancreatic cancer beyond the research setting.

5 Conclusion

There has been considerable progress in both FPC and HP and screening over the past few years. The HP mutations are better characterised than ever, although there is more work to be done on the very rare *PRSS1* mutations and the mutations associated with, but not causing pancreatitis. The computer model can now put the first ever individualised value on cancer risk for individuals from high risk groups. Potential future developments include the detection of the first screening cancer and in the longer term, the characterisation of the pre-malignant pathway and clarification of the optimal time for surgical intervention.

6 Appendix

6.1 The Patient Information Sheet for the EUROPAC FPC Secondary Screening Study

EUROPAC

The European Registry of Hereditary Pancreatic Diseases

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Patient Information Sheet

SECONDARY SCREENING IN FAMILIAL PANCREATIC CANCER

Part One

We would like to invite you to take part in a research study. Before you decide, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.
This information sheet is split into two parts. Part one will tell you the purpose of the study and what will happen to you if you take part. If you are interested in the study, part two gives more detailed background information. Please contact the EUROPAC office using the details above if you have any questions or would like to clarify anything. Take time to think everything through before taking a decision.

Key Facts

- This is a research study to see if it is possible to detect early pancreatic cancer in individuals from families that have a risk that is greater than the general population.
- The best chance of long term cure from pancreatic cancer is through early diagnosis.
- There is no single test that can detect early pancreatic cancer.
- The aim is to combine several methods so that the overall screening process will be effective.
- You can choose to have any of the available tests performed and choose not to have others.
- There is no guarantee that the process will detect early cancers or if a cancer were found whether it would be early enough to cure it.
- You can change your mind at any point and deciding not to be screened does not effect your ongoing involvement with the registry.

1.1 What is the purpose of study?

The aim of this study is to detect pancreatic cancer either as it emerges or as soon as possible after it emerges. This may increase the chance of a cure. Pancreatic screening is difficult. There is no single test that will detect every emerging cancer. The hope is to combine a number of tests to increase the overall accuracy of the screen.

1.2 Why have I been invited?

You have been invited to participate in this trial as you are already registered with the EUROPAC study. It is rare to have a genetic cause for pancreatic cancer but the information you provided when you joined the study shows that this is a possibility in your family. There are several hundred people in a similar position to you across the country.

When you registered with EUROPAC you should have had a discussion about your personal circumstances that relate to your risk of pancreatic cancer compared to the general population and had the opportunity to discuss related issues and questions with a genetic counsellor and/or a consultant pancreatic specialist. Your family history indicates that you may have an increased risk.

1.3 Do I have to take part?

It is up to you to decide. We will describe the study, go through the information sheet and leave it with you as your copy. We will ask you to sign a consent form to show that you have agreed to take part. Secondary screening is a separate study to the registry that you already take part in. If you choose not to become involved with the screening study, it does not affect your position with the registry in any way. If you wish to participate in the screening study, you can take up any or all of the proposed screening tools. If you become involved you can change your mind at any time without giving a reason. This will not affect the standard of care you receive in any way.

1.4 What will happen if I take part?

The first step in taking part in the screening study is to see a specialist in an appropriate pancreatic clinic. If you are not under a specialist or your specialist is not involved with the screening study, you will need to be referred to a specialist that is. This would normally be via your GP. The EUROPAC office will be able to provide contact details of the most appropriate clinician for you to be referred to. The clinic appointment will be for a discussion about the screening process and to check that there are no reasons why you should not take part.

If you decide to go ahead, a set of baseline investigations will be arranged. Blood tests, scans and an analysis of your pancreatic juice are available. You can choose to take up any or all of the options. They will be described in detail in the section 1.7. Depending on the results, the tests will either be repeated on an annual or three yearly basis.

You would normally be followed up in the clinic once a year. This visit would last 10-20 minutes and comprise of a review of your case, a discussion between you and your specialist, an examination if appropriate and your next investigations would be arranged. This follow up in the clinic is likely to continue at the end of the trial. If you chose to take up blood testing this would normally be conducted at the outpatient clinic.

The imaging tests and the procedure to gather your pancreatic juice would require you to attend a hospital on separate occasions. The imaging is likely to take an hour or two and you would be able to go home afterwards. An ERCP may require an overnight stay.

This is a long term project and will last for ten years, the blood tests and imaging can all be described as best medical management in high risk patients. The collection of pancreatic juice for molecular analysis is a research investigation. The findings are convincing within a laboratory setting but the molecular analysis has not been proven in living subjects in an ongoing trial.

There is no aspect of normal medical treatment that will be withheld as part of this trial and at the end of the trial it is expected that the results will be published in the scientific literature. You would not be identifiable to others as a result of any publications.

1.5 Expenses and Payments

There are no funds available for payments to those participating in this study.

1.6 What will I have to do?

The first step is to come to the clinic. If you decide to go ahead, the next step will be the blood and imaging tests. One of the blood tests (CA19-9) will normally be performed after the clinic but if the clinic is in the afternoon, the fasting glucose may require you to have a second sample taken as it is important not to have consumed any solids or any liquids other than water. This could be taken on a separate visit to the hospital or by staff at your GP practice.

The imaging tests Computed Tomography (CT) and Endoluminal Ultrasound (EUS) will require a visit to hospital. These will be at your nearest regional pancreatic centre and you would not need to stay overnight. The CT takes 10-15 minutes. You would need to lie still on a trolley whilst you move in and out of the machine but the scan does not hurt and it is rare to feel claustrophobic with modern scanners.

The EUS would not require you to stay overnight at the hospital. Again, it would be performed at your nearest pancreatic centre. The procedure will be described in the section 1.7.

Endoscopic Retrograde Cholangio-Pancreatography (ERCP) is used for collection of pancreatic juice. This is a similar type of test to the EUS and would be performed at the same centre. The purpose is to take a sample of your pancreatic juice rather than to gain an image of the pancreas. You would need sedation for this and would need either a couple of hours afterwards to rest or perhaps to stay in overnight.

If you decide to take up blood tests only, these would be repeated on an annual basis. If you opted for blood tests and imaging this would again be annual. If you had an ERCP and the DNA in your pancreatic juice was normal, we would repeat your investigations within a three year cycle, or if changes were detected the imaging would be annual. If you are having annual testing, typically, you would be asked to attend one outpatient clinic per year and have one investigation.

1.7 What is being tested?

None of the blood tests or imaging techniques are ideal. The aim is to see what combination of tests gives the best chance of picking up an early cancer and the least chance of missing one.

The methods that can be taken up include:

Blood Tests Fasting Glucose
CA19-9

Imaging Endoluminal Ultrasound (EUS)
Computed Tomography (CT)

Molecular Methods
Endoscopic Retrograde Cholangio-Pancreatography (ERCP) to allow molecular analysis of pancreatic juice

Fasting glucose is a simple test to check the sugar level in your blood. It increases if you become diabetic.

The CA19-9 is a marker that can be detected in your blood. It may increase if a pancreatic cancer is present, but also may not. It can become raised when there is no cancer present so is an incomplete test.

The EUS is a telescope test where a camera is inserted through your mouth and down your gullet into your stomach. Once inside the stomach, ultrasound images of your pancreas are obtained. As the organs are so close a very high definition picture can be obtained.

The CT is a scan that combines conventional X-ray technology and a computer to build up a three dimensional image of your insides.

The ERCP is similar to the EUS but the scope is passed a little further and a small plastic tube is used to take juice from the site where your pancreas drains into your small intestine. Changes in your pancreatic juice DNA (the genetic material that has come from the cells in your pancreas) could signify a possible early tumour that cannot be seen on the imaging investigations. These DNA changes (if found) will have arisen during your lifetime and will not be the sort of DNA changes that can be passed on to your children.

If the investigations suggested a possible problem, then your case would be discussed at a special meeting of pancreatic experts to decide on a plan of treatment. They may recommend continued surveillance, further tests to clarify the findings or even an operation if a growth were present that could be removed.

1.8 What are the alternatives?

You can take up any or all of the screening investigations. An alternative is not to participate in the screening study and continue as you have done until today.

1.9 What are the possible disadvantages to taking part?

All the screening methods have advantages and disadvantages.

The blood tests are very low risk and a serious complication would be very rare. All the equipment that would be used will be sterile, single use equipment in common use within the NHS. A bruise would be a relatively common complication.

A CT scan builds up an image by passing radiation through the body. It is known that moderate and high doses of radiation are damaging and at worst could even cause a cancer. The risk of cancer from each CT scan is described as low and calculated at somewhere between a one in a thousand and one in ten thousand chance. A single CT scan of the abdomen has been calculated to be the equivalent of a few years of normal background radiation. Your baseline imaging is likely to show that you have a normal pancreas. In this case EUS would be the main method of imaging for you. This involves no radiation and your total radiation exposure over the course of the study would therefore be limited.

The EUS involves the insertion of a scope through the mouth, down the gullet and into the stomach, where images of the pancreas are obtained by a very high definition ultrasound. There is a very small chance of the instrument puncturing the gullet or stomach, this would be expected to occur somewhere between one in a thousand and one in ten thousand times the procedure is performed. If this did happen, depending on the site of the damage, this may require an operation and in extreme cases could be fatal.

The ERCP carries a similar risk of puncturing an organ as the EUS and involves a low dose of radiation. In addition it carries a risk of acute pancreatitis. This is inflammation of the pancreas that comes on suddenly. The exact cause is unclear but is due to irritating the pancreas when the juice is collected. Overall the rate of post-ERCP pancreatitis in the UK is 1.6%. It may be more common in people with a normal pancreas, but at present a rate cannot be calculated. Attacks of acute pancreatitis are normally mild and would necessitate a short stay in hospital whilst the associated pain and nausea settle. About every sixth attack is classed as 'severe' and there can be complications. These complications can be serious and even fatal, although the chance of this is about 1 in 5000.

Other disadvantages

You may find that having screening investigations makes you more anxious about your health and your cancer risk. This is difficult to predict. Other people find having the investigations reassuring as it helps to put their mind at rest.

Any of the blood and imaging methods could detect a problem that is unrelated to pancreatic cancer. CT scans are very effective at detecting problems within the body and there is always the possibility of finding something unexpected. If this falls within the expertise of the clinician that would oversee the screening process for you, he or she would deal with this. If appropriate, you would be referred to another specialist.

It is possible that the screening investigations could detect an apparent growth in the pancreas. Growths can be 'benign', (for example, inflammation or scarring) or could be a cancer. It is difficult to tell the difference between the two before surgery. If a growth was found and it is possible to remove it, your consultant would discuss your options with you in the clinic. An operation may be offered. There are risks to any operation and it is possible that you could have an operation only to be told after the specimen has been fully analysed that there is no cancer present.

1.10 What are the side effects of any treatment received?

Treatment for pancreatic cancer is surgery, where possible. Pancreatic surgery is a major undertaking. All operations carry a risk; approximately 1-3% of all people that present clinically with pancreatic cancer and have the cancer removed, die without leaving hospital. Individuals that have growths detected by screening would be younger and healthier than the group that present clinically but the risk remains significant. A more likely scenario is that after leaving hospital you may require enzyme supplements and insulin for life.

1.11 Ionising radiation

The implications of repeated CT scanning and ERCP have been described in section 1.9.

1.12 Harm to the unborn child

Men

Damage to male sperm as a result of this study should not occur as any radiation exposure would affect the upper part of the abdomen, well away from the genital organs.

Women

It is possible that the radiation exposure involved with this study could damage an unborn child. If there is any doubt about possible pregnancy you may be asked to have a pregnancy test. Women who could become pregnant must use an effective form of contraception during the course of this study. Any woman that becomes pregnant during the course of the study should immediately inform the EUROPAC office.

1.13 What are the possible benefits?

Your family history suggests an increased risk of developing pancreatic cancer. If you developed one during the period of screening, it is hoped that this would be detected and earlier surgical treatment may achieve a cure.

1.14 What happens when the research stops?

This depends on the outcome of the study. After the end of the term of the study (ten years), if an effective screening protocol has been developed, it is likely to continue, funded by the NHS. If the proposed investigations are shown to be ineffective at accurately diagnosing early pancreatic cancer at a time when it can be cured, screening would be unlikely to continue to be offered in its present form through the NHS. Individual consultants may continue to offer some ongoing surveillance via the NHS within families with an elevated risk, depending on their own personal practice.

1.15 What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. More detailed information on this will be covered in part two.

1.16 Will my taking part be confidential?

Yes, we will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in part two.

If the information in part one has interested you and you are considering participating in the study, please read the additional information in part two before making a decision.

Part Two

2.1 What if new relevant information becomes available?

New information about pancreatic cancer or the screening investigations employed may become available during the course of the study. If this happens, your research doctor will tell you and discuss whether you should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your ongoing care to continue. If you decide to continue in the study he/she may ask you to sign an updated consent form.

It is possible that new information might become available that would prompt your research doctor to advise you to consider withdrawing from the study. He/she will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, we will tell you and arrange your ongoing care.

2.2 What will happen if I don't want to carry on with the study?

If you want to withdraw from the study, please contact the staff in the EUROPAC office and tell the consultant who has been overseeing your screening investigations. If you would like the EUROPAC office staff to do this for you, they will be happy to do this. If you withdraw from the screening study we would be keen for you to remain involved with the registry and let us know your progress. Withdrawal from the study would not affect your clinical care. Information we have collected before your withdrawal will not be destroyed or erased but will not be considered when the screening process is analysed.

If you wish to withdraw any stored blood or tissue samples, we will do this wherever possible. Sometimes samples will have been combined with others. If all the material that you originally donated cannot be destroyed, all reference to you will be removed from any database relating to the sample. This means that the sample will be anonymised and will not be traceable back to you under any circumstances.

2.3 What if there is a problem?

Complaints

If you have a concern about any aspect of this study, you should ask to speak to the staff in the EUROPAC office who will do their best to answer your questions. Contact details for EUROPAC are given on the front of this information sheet. The telephone number is 0151 706 4168. If you would rather not deal with the staff in the office, the clinical consultant overseeing your care or your General Practitioner (GP) should be contacted. If you do not feel that either of these routes is open to you, you could

complain to the chief executive of the trust where you are being screened and the normal NHS complaints procedure would be followed.

Harm

If you were to come to any harm as a result of this study, NHS indemnity and insurance procedures apply. In the event that something does go wrong and you are harmed during the research and this is due to negligence, you may have grounds for a legal action for compensation against the University of Liverpool and the Royal Liverpool and Broadgreen University Hospital Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. NHS Indemnity does not offer compensation for harm that is not the result of negligence.

NHS based research

NHS bodies are liable for clinical negligence and other negligent harm to individuals covered by their duty of care. NHS Institutions employing researchers are liable for negligent harm caused by the design of studies they initiate.

2.4 Will taking part be confidential?

All data handling and storage will be in accordance with Caldicott principles and/or the Data Protection Act 1998. All information which is collected about you during the course of the research will be kept strictly confidential and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised. If you participate, you have the right to check the accuracy of data held about you is correct. Data is collected from hospital computer systems and casenotes and is stored on the main EUROPAC registry database, which is password protected and isolated from the internet. Only the lead scientist, the EUROPAC database manager and the EUROPAC research fellow have the passwords to access the data. The data will be used to allow statistical analysis for any detectable changes in patients that both develop and do not develop pancreatic cancer. After the period of this study, data may be retained, subject to further ethical committee approval. Any destruction of data will be performed in a secure fashion.

2.5 Involvement of the General Practitioner?

Results would normally be communicated to your GP unless you explicitly state at the appropriate point on the consent form that they should not be. As a general principle, it is important that your GP has as much information that relates to your healthcare as possible. This enables them to make accurate decisions relating to your health.

2.6 What will happen to any samples that I give?

The screening blood tests that are taken will be processed and blood will be stored for less than a month afterwards before being destroyed in accordance with NHS procedures. Pancreatic juice gathered at ERCP will be stored in the Division of Surgery & Oncology at Liverpool University for ten years. If changes were detected in your pancreatic juice you may be asked for a further blood sample for research, which will be stored for a maximum of 10 years. This blood sample would be used to see if the changes detected in your pancreatic juice could be found in your blood. It is very unlikely that this will have any immediate consequence for you, but it may allow us to develop improved screening systems that could be applied later. If you were ever to require an operation we would ask for your permission to retain small amounts of tissue.

Any tissue or blood storage complies with both the data protection act and the tissue act. Information is coded and kept isolated from the internet on password protected databases. Destruction of any data will be performed in a secure fashion.

2.7 Will any genetic tests be done?

There will be no genetic (inheritable) testing done as part of this study. If genetic tests are available to you this will have been discussed as part of your existing membership of EUROPAC. Your eligibility for genetic testing in future will not be affected by your inclusion in the screening study and any future discussions between you and members of the EUROPAC study group regarding genetic testing will be completely independent of the screening study.

The molecular analysis of the DNA in your pancreatic juice is to look for cancer related changes; detection of these changes will have no implications for the health of your children or other relatives. As these changes will not have been inherited and cannot be passed on to your children, tests for these changes would not normally be described as genetic tests.

2.8 What will happen to the results of this study?

You will be made aware of the results of your screening blood and imaging investigations during the course of the study by your specialist, normally in the outpatient clinic. Any results from the molecular analysis of your pancreatic juice would only be given to you if you expressly request them. Although these tests may be used by your specialist to guide the timing of the other investigations, the significance of these test results are not clear at present and they should be considered as research data.

The overall results of the research project will be made available at the end of the study (in an anonymised form) via publications in scientific journals and presentations at scientific meetings. Once results have been published, copies will be available from the EUROPAC office and anonymous non-sensitive information may be made available on the EUROPAC website.

2.9 Who is organising and funding the research?

The sponsors of this study are The Royal Liverpool University Hospital and The University of Liverpool. The study is being funded by the European Union (EU) and Cancer Research UK (CRUK). The research team do not have any financial interest in the study and there are no financial payments or inducements of any kind for recruiting participants to the study.

2.10 Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Warwickshire Research Ethics Committee. The study was also funded only after review by the EU and Cancer Research UK.

This copy of the information sheet and a signed consent form will be yours to keep. This is both for your own records and in case of any queries or concerns in the future.

2.11 Further Information and contact details

Thank you for taking the time to read this information sheet. Take time to think through the issues raised. If you have any questions please contact the EUROPAC office and we will be happy to discuss things with you.
Whenever you are in contact with the EUROPAC office, your contact is:

.....

If you would like to gather information from other sources, you may find these resources of interest.

General information about research.
The Medical Research Council: http://www.ctu.mrc.ac.uk/default.asp
Cancer Research UK: http://www.cancerhelp.org.uk/default.asp

Specific information about this research project.
Contact the EUROPAC office (0151 706 4168)

Advice as to whether you should participate.
Contact the EUROPAC office (0151 706 4168)
You could discuss the issues with your GP

Contact details in the case of any problem.
The EUROPAC office (0151 706 4168)
The pancreas specialist that is arranging your screening.

6.2 The Consent Form for the EUROPAC FPC Secondary Screening Study

Modelling of risk in individuals with a possible genetic predisposition for pancreatic cancer

EUROPAC

The European Registry of Hereditary Pancreatic Diseases

EUROPAC Study Co-ordinator, 5th Floor UCD Building, Royal Liverpool University Hospital, Daulby Street, Liverpool, L69 3GA, UK

Tel: +44 151 706 4168 europac@liv.ac.uk www.europac-org.eu Fax: +44 151 706 5826

Consent Form

Secondary Screening in Familial Pancreatic Cancer

MREC Reference 07/H1211/96

EUROPAC Unique identification Number

PLEASE INITIAL BOX

1. I confirm that I have read and understand the information sheet dated 8th September 2007 (version 2) for the above study. I have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected and without affecting my continued involvement with the EUROPAC registry. ☐

3. I have had the screening study explained to me and understand the timing of proposed investigations. ☐

4. I have had the advantages and disadvantages of each part of the screening study discussed with me. ☐

5. I understand that I can choose to have any combination of the tests offered. ☐

6. I agree/I do not agree* to have a blood sample taken and analysed for the presence of CA 19-9 (*please delete as appropriate) ☐

7. I agree/I do not agree* to have a blood sample taken and analysed to measure fasting blood Glucose (*please delete as appropriate) ☐

8. I agree/I do not agree* to Endoluminal Ultrasound (EUS) if indicated in the screening process (*please delete as appropriate) ☐

9. I agree/I do not agree* to Computerised Tomography (CT) scanning if indicated in the screening process (*please delete as appropriate) ☐

10. I agree/I do not agree* to Pancreatic Juice collection via Endoscopic Retrograde Cholangio-Pancreatography (ERCP) if indicated in the screening process (*please delete as appropriate) ☐

11. I agree/I do not agree* that a blood sample may also be used for molecular testing (see section 2.6 and 2.7 of the information sheet dated 8th September 2007, version 2). (*please delete as appropriate) ☐

12. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the EUROPAC study group; from regulatory authorities; or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

13. I understand that the results of the ongoing trial will be collated on a database. All data will be stored in accordance with the Data Protection Act 1998. ☐

14. I understand that anonymous data from this screening may be used for medical and scientific purposes and that it is intended to publish the findings in the scientific literature. ☐

15. I agree to my GP being informed of my participation in the study. ☐

16. I agree to my GP being informed of significant clinical findings that may arise from the study. ☐

17. I understand that this is a research study and is not proven as a screen for detecting pancreatic cancer. ☐

18. I agree to take part in the secondary screening study. I understand that I can change the consents recorded on this form at any point in the future. ☐

Name of Patient	Signature	Date
Name of Person taking Consent	Signature	Date

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

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